



(51) International Patent Classification:

A61K 38/19 (2006.01) A61K 47/42 (2017.01)
A61K 9/00 (2006.01)

(21) International Application Number:

PCT/US2017/043075

(22) International Filing Date:

20 July 2017 (20.07.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/365,189 21 July 2016 (21.07.2016) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US).

(72) Inventors: RYAN, Allen F.; 502 Pacific Avenue, Solana Beach, California 92075 (US). TAURA, Akiko; Kyoto University Hospital, Department of Otolaryngology, Head and Neck Surgery, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507 (JP).

(74) Agent: WAHLSTEN, Jennifer L. et al.; WEAVER AUSTIN VILLENEUVE & SAMPSON LLP, P.O. Box 70250, Oakland, California 94612-0250 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: METHODS FOR INDUCING STEREOCILIA ON HAIR CELLS

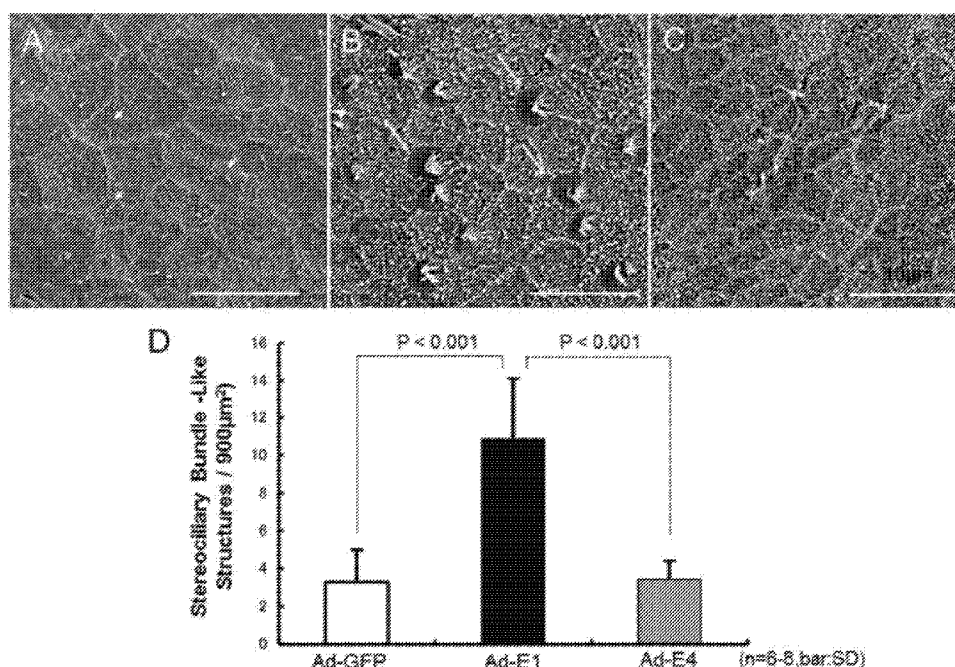


Fig. 6A-D

(57) Abstract: Provided are methods for inducing functional stereocilia and stereocilia bundles in inner ear auditory hair cells comprising administering to and/or expressing in the hair cells sufficient levels of espin isoform 1 (ESPN1). Further provided are otic solutions for delivering a polynucleotide encoding ESPN1 or a ESPN1 polypeptide to the inner ear of a subject.

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))*

METHODS FOR INDUCING STEREOCILIA ON HAIR CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) of
5 U.S. Provisional Application No. 62/365,189, filed on July 21, 2016, which is hereby
incorporated herein in its entirety for all purposes.

STATEMENT OF GOVERNMENTAL SUPPORT

[0002] This invention was made with government support under Grant No
DC000129 awarded by the National Institutes of Health, and Grant No. BX001295 awarded
10 by the United States Department of Veterans Affairs. The government has certain rights in
the invention.

BACKGROUND

[0003] Hearing and balance disorders are important causes of disability in patients.
The major cause is sensory hair cell (HC) loss in the inner ear (1). In the damaged sensory
15 epithelia of birds, new HCs regenerate in both the vestibular (2) and auditory sense organs
(3). However, in mammals, HC loss is irreversible. For this reason, the regeneration of
HCs in mammals is a subject of considerable research. Successful regeneration of
functional HCs in mammals would be a major advance in the therapy of inner ear disorders.

[0004] When HCs are damaged, apical structures including stereociliary bundles are
20 often the first cellular elements to be lost (4). The stereociliary bundle, as the site of
mechanotransduction, is critical for HCs to maintain their physiological function. Some of
these bundleless HCs can survive for considerable periods of time, but spontaneous
stereociliary regeneration is not normally observed (5). So the replacement of stereocilia on
surviving HCs is a potential strategy for functional recovery following inner ear sensory cell
25 damage.

[0005] The espins are a family of actin bundling proteins, produced in multiple
isoforms from a single gene (espn). They are localized in the stereocilia of HCs as well as
the microvilli of many sensory cells (6). Espins are associated with the parallel actin
bundles of stereocilia throughout stereocilia formation during development (7-8).
30 Stereocilia are hypoplastic in the absence of the espn gene, exhibiting reduced length and

abnormal structure. This results in hearing loss and vestibular dysfunction in the Jerker mouse model (9) and in the human DFNB36 deafness mutation (10). Given its critical role in stereociliary development, we reasoned that induced espin expression might be useful for stereociliary bundle regeneration.

5 **[0006]** Gene therapy has been found to be a promising tool for application to the inner ear. The labyrinth is anatomically well suited to local gene therapy, because it is a closed system that is isolated from other organs and is relatively easy to access via the middle ear, thus allowing local application and relative isolation of viral vectors with minimal spread to other sites (11). Recently, a degree of success has been reported using
10 experimental gene therapy for various types of inner ear disorder caused by ototoxic drugs and genetic abnormalities (12-14).

[0007] With respect to HC regeneration, techniques which recapitulate the developmental program of HCs have generally been employed. These can, under the appropriate circumstances, induce nonsensory cells to convert to HCs with or without cell
15 division (15-19). One of the earliest steps in HC specification is induction of the basic helix-loop-helix transcription factor ATOH1 by EYA1 and SIX1 (20). ATOH1 interacts with the Notch signaling system to form the mosaic of HCs and supporting cells, with Notch ligands suppressing the production of ATOH1 in the latter (21). The transduction
20 inner ear supporting cells with the *atoh1* gene has been reported to be effective in inducing HC formation, especially in developing mammals (22). Blockade of Notch signaling using γ -secretase inhibitors has also been shown to be effective in mammalian HC induction, since Notch signals act on supporting cells to inhibit their differentiation into HCs. Several studies have reported the conversion of supporting cells into HCs following Notch
25 inhibition (23-25). The transdifferentiation process in the neonatal utricle is characterized by both mitotic and non-mitotic processes (21,26). Loss of HCs induces generally modest spontaneous regeneration due to loss of Notch inhibition of supporting cells. Notch inhibitors greatly enhance this process, inducing some supporting cells to dedifferentiate, enter the cell cycle, and produce daughter cells which can assume either a supporting cell or HC phenotype. Other supporting cells de-differentiate and transdifferentiate directly into
30 HCs. However, in many cases regenerated HCs are immature and exhibit no or only partial function (23,25,27).

[0008] These prior studies suggest that enhancing the growth of stereocilia may be a useful means by which to restore cochlear function following HC damage and/or regeneration.

SUMMARY

5 [0009] In one aspect, provided are methods of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell in a subject in need thereof. In varying embodiments, the methods comprise administering to and expressing within the hair cell in the inner ear of the subject a polynucleotide encoding espin isoform 1 (ESPN1), espin isoform 2 (ESPN2) and/or espin isoform 3 (ESPN3). In varying embodiments, the
 10 subject is a mammal, *e.g.*, a human, a non-human primate, a canine, a feline, an equine or a rodent (a mouse, a rat, a hamster, a guinea pig, a rabbit). In a further aspect, provided are methods of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell comprising administering to and expressing within the hair cell a polynucleotide encoding espin isoform 1 (ESPN1), espin isoform 2 (ESPN2) and/or espin isoform 3 (ESPN3). In
 15 varying embodiments, the hair cell is *in vivo*. In varying embodiments, the hair cell is *in vitro*. In varying embodiments, the hair cell is a damaged or regenerated hair cell. In some embodiments, the hair cell has been damaged by exposure to an aminoglycoside, *e.g.*, gentamicin. In varying embodiments, the polynucleotide encoding ESPN1, ESPN2 and/or ESPN3 is expressed under the control of a promoter heterologous to the polynucleotide,
 20 *e.g.*, a constitutive promoter or an inducible promoter. In varying embodiments, the polynucleotide encoding ESPN1 is administered to the hair cell in a viral vector, *e.g.*, a viral vector selected from the group consisting of adenovirus, adeno-associated virus, lentivirus, and retrovirus. In varying embodiments, the viral vector is replication defective. In varying embodiments, the polynucleotide encoding ESPN1 is administered to the hair cell in a
 25 plasmid vector. In some embodiments, the polynucleotide encoding ESPN1 has at least 60% sequence identity, *e.g.*, at least about 65%, 70%, 75%, 80%, 85%, 90%, 95%, 95%, 97%, 98%, 99% or 100% sequence identity, to one or more of SEQ ID NOs: 1, 3, 5, 7 or 9. In varying embodiments, the methods further comprise administering to the hair cell a polynucleotide encoding ATOH1 (atonal bHLH transcription factor 1) or an ATOH1
 30 polypeptide. In varying embodiments, the methods further comprising administering to the hair cell a Notch inhibitor, *e.g.*, a γ -secretase inhibitor. In varying embodiments, the γ -secretase inhibitor is DAPT (N-[N-[(3,5-Difluorophenyl)acetyl]-L-alanyl]-L-phenylglycine-tert-butyl). In varying embodiments, the induced stereociliary bundles

comprise a stair-step arrangement and a single, central kinocilium. In varying embodiments, the induced stereocilia in an induced stereociliary bundle are linked at their tops. In varying embodiments, the induced stereociliary bundles exhibit functional mechanoelectrical transduction channels.

- 5 **[0010]** In a further aspect, provided are methods of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell in a subject in need thereof comprising administering to the hair cell in the inner ear an ESPN1, ESPN2 and/or ESPN3 polypeptide. In varying embodiments, the subject is a mammal, *e.g.*, a human, a non-human primate, a canine, a feline, an equine or a rodent (a mouse, a rat, a hamster, a guinea pig, a rabbit). In another aspect, provided are methods of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell. In some embodiments, the methods comprise administering to the hair cell an ESPN1, ESPN2 and/or ESPN3 polypeptide. In varying embodiments, the hair cell is *in vivo*. In varying embodiments, the hair cell is *in vitro*. In varying embodiments, the hair cell is a damaged or regenerated hair cell.
- 10 In varying embodiments, the hair cell has been damaged by exposure to an aminoglycoside, *e.g.*, gentamicin. In varying embodiments, the ESPN1 polypeptide is administered in a nanoparticle, a liposome and/or a microparticle. In varying embodiments, the ESPN1 polypeptide has at least 60% sequence identity, *e.g.*, at least about 65%, 70%, 75%, 80%, 85%, 90%, 95%, 95%, 97%, 98%, 99% or 100% sequence identity, to one or more of SEQ
- 15 ID NOs: 2, 4, 6, 8 or 10. In some embodiments, the methods further comprise administering to the hair cell a polynucleotide encoding ATOH1 (atonal bHLH transcription factor 1) or an ATOH1 polypeptide. In varying embodiments, the methods further comprising administering to the hair cell a Notch inhibitor, *e.g.*, a γ -secretase inhibitor. In varying embodiments, the γ -secretase inhibitor is DAPT (N-[N-[(3,5-
- 20 Difluorophenyl)acetyl]-L-alanyl]-L-phenylglycine-tert-butyl). In varying embodiments, the induced stereociliary bundles comprise a stair-step arrangement and a single, central kinocilium. In varying embodiments, the induced stereocilia in an induced stereociliary bundle are linked at their tops. In varying embodiments, the induced stereociliary bundles exhibit functional mechanoelectrical transduction channels.
- 25 **[0011]** In a further aspect, provided is an otic solution (*e.g.*, a solution formulated for delivery to the inner ear) comprising a polynucleotide encoding espin isoform 1 (ESPN1), espin isoform 2 (ESPN2) and/or espin isoform 3 (ESPN3). In varying embodiments, the polynucleotide is in a viral vector or a plasmid vector, *e.g.*, a viral vector
- 30

selected from the group consisting of adenovirus, adeno-associated virus, lentivirus, and retrovirus. In varying embodiments, the viral vector is replication defective. In varying embodiments, the polynucleotide encoding ESPN1 has at least 60% sequence identity, *e.g.*, at least about 65%, 70%, 75%, 80%, 85%, 90%, 95%, 95%, 97%, 98%, 99% or 100% sequence identity, to one or more of SEQ ID NOs: 1, 3, 5, 7 or 9.

[0012] In a further aspect, provided is an otic solution (*e.g.*, a solution formulated for delivery to the inner ear) comprising an ESPN1, ESPN2 and/or ESPN3 polypeptide. In varying embodiments, the ESPN1 polypeptide has at least 60% sequence identity to one or more of SEQ ID NOs: 2, 4, 6, 8 or 10.

10 **DEFINITIONS**

[0013] A “polynucleotide” is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5’ to the 3’ end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated “bp”), nucleotides (“nt”), or kilobases (“kb”). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term “base pairs”. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired.

[0014] The terms “identical” or percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, share at least about 80% identity, for example, at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity over a specified region to a reference sequence, *e.g.*, an ESPN polynucleotide or polypeptide sequence as described herein, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences are then said to be “substantially identical.” This definition also refers to the complement of a test sequence.

Preferably, the identity exists over a region that is at least about 25 amino acids or nucleotides in length, for example, over a region that is 50, 100, 200, 300, 400, 500, 600, 700, 800 amino acids or nucleotides in length, or over the full-length of a reference sequence.

5 **[0015]** For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be
10 designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. For sequence comparison of nucleic acids and proteins to ESPN nucleic acids and proteins, the BLAST 2.4.0+ algorithms and the default parameters are used.

[0016] A “comparison window”, as used herein, includes reference to a segment of
15 any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison
20 can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat’l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software
25 Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Ausubel et al., eds., *Current Protocols in Molecular Biology* (1995 supplement)). Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST
30 2.0 algorithms, which are described in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990) and Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1977), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (on the worldwide web at ncbi.nlm.nih.gov/).

[0017] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

[0018] The terms "patient," "subject," "individual" interchangeably refer to a mammal, for example, a human or a non-human primate, a domesticated mammal (*e.g.*, a canine or feline), an agricultural mammal (*e.g.*, a bovine, porcine, ovine, equine), a laboratory mammal (a mouse, rat, hamster, guinea pig, rabbit).

[0019] The term "co-administer" refers to the simultaneous presence of two active agents in the blood of an individual. Active agents that are co-administered can be concurrently or sequentially delivered.

[0020] The phrase "cause to be administered" refers to the actions taken by a medical professional (*e.g.*, a physician), or a person controlling medical care of a subject, that control and/or permit the administration of the agent(s)/compound(s) at issue to the subject. Causing to be administered can involve diagnosis and/or determination of an appropriate therapeutic or prophylactic regimen, and/or prescribing particular agent(s)/compounds for a subject. Such prescribing can include, for example, drafting a prescription form, annotating a medical record, and the like.

[0021] The terms "effective amount" or "amount effective to" or "therapeutically effective amount" includes reference to a dosage of a therapeutic agent sufficient to produce a desired result, such as inhibiting, reducing or preventing the disease condition sought to be inhibited, ameliorated and/or treated, *e.g.*, hearing loss due to loss of functional stereocilia and/or stereociliary bundles on auditory hair cells, *e.g.*, due to presbycusis (age-related), ototoxicity, noise induced hearing loss, viral infections of the inner ear, autoimmune inner ear diseases, genetics/heredity, inner ear barotrauma; physical trauma, surgical trauma, and/or inflammation. The term "effective amount" as used in relation to pharmaceutical compositions, typically refers to the amount of the active ingredient, *e.g.* the peptides of the

invention, which are required to achieve the desired goal. For example, in therapeutic applications, an effective amount will be the amount required to be administered to a patient to result in treatment of the particular disorder for which treatment is sought (*e.g.*, hearing loss due to loss of functional stereocilia and/or stereociliary bundles on auditory hair cells, *e.g.*, due to presbycusis (age-related), ototoxicity, noise induced hearing loss, viral infections of the inner ear, autoimmune inner ear diseases, genetics/heredity, inner ear barotrauma; physical trauma, surgical trauma, and/or inflammation). The term "treatment of a disorder" denotes the reduction or elimination of symptoms of a particular disorder. Effective amounts will typically vary depending upon the nature of the disorder, the peptides used, the mode of administration, and the size and health of the patient. In one embodiment, the effective amount of the peptides of the invention ranges from 1 μ g to 1 g of peptide for a 70 kg patient, and in one embodiment, from 1 μ g to 10 mg. In one embodiment, the concentration ESPN polynucleotide or polypeptide administered ranges from 0.1 μ M to 10 mM, and in one embodiment, from 5 μ M to 1 mM, in one embodiment, from 5 μ M to 100 μ M, and in one embodiment from 5 μ M to 40 μ M.

[0022] As used herein, the term "treating" is intended to mean the administration of a therapeutically effective amount of an ESPN polynucleotide and/or ESPN polypeptide described herein to a subject who is experiencing loss or impairment of hearing, loss or impairment of balance, or injury to or loss of vestibular hair cells, neurons, supporting cells, or dark cells, in order to minimize, reduce, or completely prevent or restore, the loss of hearing, the loss of balance function or of hair cells, neurons or dark cells of the vestibular portion of the inner ear. Treatment is intended to also include the possibility of inducing, causing or facilitating regeneration of the cellular elements of the inner ear including hair cells, supporting cells, dark cells, neurons and subcellular organelles of these cells including, synapses, stereocilia bundles, kinocilia, mitochondria and other cell organelles, or mechanical and functional supporting structures such as otoconia, cupula and crista of the inner ear. Treatment is also intended to prevent recurrent degeneration after regeneration of cellular elements of the inner ear, including hair cells, supporting cells, dark cells, neurons and subcellular organelles of these cells including synapses, stereocilia bundles, kinocilia, mitochondria and other cell organelles, or mechanical and functional supporting structures such as otoconia, cupula and crista of the inner ear. Treatment is also intended to mean the partial or complete restoration of hearing or balance function regardless of the cellular mechanisms involved.

[0023] As used herein, "loss of balance" or "impairment to the sense balance", "impaired balance", "loss of balance function" and "balance disorders" are terms that are intended to refer to a deficit in the vestibular system including associated neural structures, or vestibular function of a subject compared to the system of a normally functioning human.

5 This deficit may completely or partially impair a subject's ability to maintain posture, spatial orientation, locomotion and any other functions associated with normal vestibular function. Balance disorders also include intermittent attacks of vertigo, such as those seen in Meniere's Disease, or other inner ear disorders.

[0024] As used herein, the term "administration" is intended to include, but is not
10 limited to, the following delivery methods: topical, including topical delivery to the round window membrane of the cochlea, oral, parenteral, subcutaneous, transdermal, and transbuccal administration. In one example, the permeability of the round window membrane is enhanced by partially digesting it using a protease prior to transfection of the inner ear cells with an ESPN polynucleotide and/or ESPN polypeptide described herein.

15 **[0025]** As used herein the term "hearing loss" is intended to mean any reduction in a subject's ability to detect sound. Hearing loss is defined as a 10 decibel (dB) standard threshold shift or greater in hearing sensitivity for two of 6 frequencies ranging from 0.5-6.0 (0.5, 1, 2, 3, 4, and 6) kHz (cited in Dobie, R. A. (2005) Audiometric Threshold Shift Definitions: Simulations and Suggestions, Ear and Hearing 26(1) 62-77). Hearing loss can
20 also be only high frequency, and in this case would be defined as 5 dB hearing loss at two adjacent high frequencies (2-6 kHz), or 10 dB at any frequency above 2 kHz. One example of hearing loss is age-related (or aging-related) hearing loss, which is the gradual onset of hearing loss with increasing age.

[0026] As used herein, the term "prevention", in the context of the loss of or
25 impairments to the sense of balance, death or injury of vestibular hair cells, death or injury of vestibular neurons, injury to functionally important mechanical structures such as the ototoconia or cupula, death or injury of vestibular dark cells and the like refers to minimizing, reducing, or completely eliminating the loss or impairment of balance function or damage, death or loss of those cells through the administration of an effective amount of
30 one of the vectors described herein, ideally before an oxidatively stressful insult, or less ideally, shortly thereafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Figures 1A-D illustrate the number of stereociliary bundles and HC bodies after GM treatment. A: control (myosin7A), B: control (phalloidin), C: GM 10d AT (myosin7A), D: GM 10d AT (phalloidin). After GM treatment, almost all hair bundles are lost, and the number of HC bodies decreases. However, some HCs without hair bundles remain even 10 days after damage (n=5 explants/time). Points represent means and bars represent SD (standard deviation).

[0028] Figures 2A-C illustrate adenoviral transduction after GM treatment. A: After Ad-GFP transduction, GFP expression was diffused throughout the cell body. B: After Ad-E1 transduction, a filamentous pattern of GFP expression was observed. C: After Ad-E4 transduction, a filamentous pattern of GFP expression was also observed. Most GFP positive cells were negative for Myosin7A (Red), suggesting that the majority of transduced cells were supporting cells. Red: Myosin7A, Green: EGFP, White: phalloidin, Blue: DAPI.

[0029] Figures 3A-C illustrate the effects of DAPT exposure after GM treatment. A, A': control explant (2 days culture). HCs are densely present. B, B': Two days after GM treatment. HCs decreased dramatically. C, C': DAPT application after GM treatment. Many myosin7A-positive cells were observed. In a tissue section, myosin7A-positive cells were numerous in the supporting cell layer (arrows). A-C: whole mount. A'-C': section. Red: Myosin7A, Blue: DAPI. HC*: HC layer SC**: supporting cell layer.

[0030] Figures 4A-C illustrate the time course of GFP expression after adenoviral transduction. A: Twenty-four hrs after Ad-E1 administration. B: Forty-eight hours after Ad-E1 administration. Filamentous GFP expression was observed (arrows). C: Intensity ratio of GFP expression for a typical explant. Adenoviral GFP expression is maximal 46 hrs after adenovirus administration.

[0031] Figures 5A-C illustrate the hair bundle-like structures on Ad-E1 transduced explants. A: Whole mount utricular explant. Many myosin7A-positive cells with hair-bundle-like structures on their apical surfaces are observed (e.g. arrows). B: Sectioned explant. Myosin7A-positive cell bodies are observed in the supporting cell layer. Hair-bundle-like structures protrude from extensions of these cells that reach the apical surface of the epithelium (e.g. arrowhead). Red: Myosin7A; Green: EGFP; Blue: DAPI. C: Number of hair-bundle-like structures observed after adenoviral transduction. In Ad-GFP transduced explants, a few damaged stereociliary bundles remained. However, in Ad-E1

transduced explants, there are many more immature hair bundles. In Ad-E4 transduced explants, some filamentous cilia-like structures are observed. The number of bundle-like structures in Ad-E1 transduced explants was significantly greater than in control (Ad-GFP) explants than in Ad-GFP transduced explants ($p < .05$, 6-7 explants per group)..

5 **[0032]** Figures 6A-D illustrate scanning electron microscopy (SEM) analysis. A-C: Representative explants. A: Ad-GFP transduction. Reticulated cell borders are observed. Some damaged hair bundles and basal bodies (e.g. arrowheads) remain, but few structures resembling stereociliary arrays are present. B: Ad-E1 transduced explants. Many apparently immature hair-bundle-like structures are observed (e.g. arrows). C: Ad-E4. Extended microvillus-like structures and microvilli are apparent. There are some damaged stereociliary bundles, but few immature hair-bundle-like structures are apparent. D. Quantitative analysis: Number of stereociliary bundle-like structures in SEM. Significantly more of these structures are observed on Ad-E1 transduced explants, than on those transduced with Ad-GFP or Ad-E4 ($P < .001$; 6-8 explants per treatment condition).

15 **[0033]** Figures 7A-B illustrate high magnification of Ad-E1 transduction in SEM observation. A: In each cell, one kinocilium is observed in the center of the cell and stereocilia are present on approximately half of the apical surface. B: Stereocilia exhibit a staircase pattern. The upper ends of the stereocilia appear to adhere to one another.

20 **[0034]** Figures 8A-D illustrate functional analysis of stereociliary bundles using FM1-43FX. A: Ad-GFP transduced explants. B: Ad-E1 transduced explants. C: Ad-E4 transduced explants. Only a few FM1-43 positive cells, as indicated by fluorescent labeling, are observed in A and C. In contrast, many more FM1-43 positive cells are seen in B (arrows). D: Quantitative FM1-43 analysis. In Ad-E1 transduced explants, there are a number of FM1-43-loaded cells. However, FM1-43 positive cells are much less prevalent in Ad-GFP and Ad-E4 explants. These differences were significant ($P < .05$, 6-9 explants per treatment condition).

DETAILED DESCRIPTION

1. Introduction

30 **[0035]** In the present study, we examined *espn* gene transduction to induce the regeneration of functional stereociliary arrays, following ototoxic HC damage and HC regeneration induced by Notch inhibition. We evaluated viral vector transduction with Espin1 or Espin4, both linked to GFP. A GFP-only vector was used as a control.

[0036] Once inner ear hair cells (HCs) are damaged by drugs, noise or aging, their apical structures including the stereociliary arrays are frequently the first cellular feature to be lost. While this can be followed by progressive loss of HC somata, a significant number of HC bodies often remain even after stereociliary loss. However, in the absence of stereocilia they are nonfunctional. HCs can sometimes be regenerated by Atoh1 transduction or Notch inhibition, but they also may lack stereociliary bundles. We therefore sought to develop methods for the regeneration of stereocilia, in order to achieve HC functional recovery. Espin is an actin bundling protein known to participate in stereociliary elongation during development. We evaluated stereociliary array regeneration in damaged vestibular sensory epithelia in tissue culture, using viral vector transduction of two espin isoforms. Utricular HCs were damaged with aminoglycosides. The utricles were then treated with a γ -secretase inhibitor, followed by espin or control transduction and histochemistry. While γ -secretase inhibition increased the number of HCs, few had stereociliary arrays. In contrast, 46 hrs after espin1 transduction, a significant increase in hair-bundle-like structures was observed. These were confirmed to be immature stereociliary arrays by scanning electron microscopy. Increased uptake of FM1-43 uptake provided evidence of stereociliary function. Espin4 transduction had no effect. The results demonstrate that espin1 gene therapy can restore stereocilia on damaged or regenerated HCs.

2. Subjects Amenable to Treatment

[0037] The methods described herein for inducing functional stereocilia and/or stereociliary bundles on inner ear auditory hair cells finds use for treating patients who have suffered hearing losses or who are likely to suffer hearing losses due to hair cell damage. In one example, the hearing loss is due to presbycusis (age-related hearing loss). In addition to age-related hearing loss, we also contemplate that other types of hearing loss may be treatable by administration to hair cells of ESPN1, ESPN2 and/or ESPN3, optionally co-administered with ATOH1. Examples of other types of hearing loss include, for example: 1) ototoxicity caused by chemical or pharmaceutical agents, for example, antineoplastic agents such as cisplatin or related compounds, aminoglycosides, antineoplastic agents, and other chemical ototoxic agents; 2) noise induced hearing loss, either from acoustic trauma or blast injury; 3) therapeutic radiation; 4) viral infections of the inner ear, such as Herpes Simplex, cytomegalovirus or other viruses or infectious agents (such as Lyme Disease) that can cause inner ear hearing loss; 5) autoimmune inner ear

diseases; 6) genetic hearing losses that may have an apoptotic component; 7) inner ear barotrauma such as diving or acute pressure changes; 8) physical trauma such as that caused by head injury, or surgical trauma from surgical intervention in the inner ear; 9) inflammation or other response to administration of other inner ear regenerative compounds or gene therapy techniques; 10) ischemic damage to the inner ear such as in vasculitis, or following acoustic neuroma surgery; and 11) idiopathic or vestibular disorders such as sudden sensorineural hearing loss.

3. Methods of Inducing Stereocilia in Hair Cells

[0038] Generally, the methods entail administering to an inner ear auditory hair cell a polynucleotide encoding Espin isoforms 1, 2 and/or 3, and/or Espin isoforms 1, 2 and/or 3 polypeptides. In some embodiments, ESPN1, ESPN2 and/or ESPN3 is co-administered with ATOH1, in polynucleotide or polypeptide form.

[0039] In general, there are two approaches to gene therapy in humans. For in vivo gene therapy, a vector encoding the gene of interest can be administered directly to the patient. Alternatively, in ex vivo gene therapy, cells are removed from the patient and treated with a vector to express the gene of interest. In the ex vivo method of gene therapy, the treated cells are then re-administered to the patient.

[0040] Numerous different methods for gene therapy are well known in the art. These methods include, but are not limited to, the use of DNA plasmid vectors as well as DNA and RNA viral vectors. In the present compositions and methods, these vectors are engineered to express ESPN isoforms 1, 2 and/or 3 (and optionally, ATOH1) when integrated into patient cells.

[0041] It is known that in the auditory system, three major viral vectors have been investigated for cochlear gene transfection: (1) lentivirus, (2) adenovirus and (3) Adeno-associated virus (AAV). The gene transfected by adenovirus vector has limited expression time and the vector has been associated with adverse immune reactions (Staecker, Brough, Praetorius, & Baker, 2004). The lentivirus vector, although capable of maintaining long term expression, is particularly suited for targeting neurons, but not hair cells (Federico, 1999). AAV vectors have several advantages such as long lasting expression of synthesized genes (Cooper et al, 2006), and low risk for pathogenic reactions (because they are artificially manufactured and not ototoxic) (Kaplitt et al., 1994). AAV vectors useful for

targeting polynucleotides to the inner ear are described, *e.g.*, in Shu, *et al.*, *Hum Gene Ther.* (2016) 27(9):687-99 and Kilpatrick, *et al.*, *Gene Ther.* (2011) 18(6):569-78.

[0042] Adenoviruses are able to transfect a wide variety of cell types, including non-dividing cells. The discovery includes the use of any one of more than 50 serotypes of
5 adenoviruses that are known in the art, including the most commonly used serotypes for gene therapy: type 2 and type 5. In order to increase the efficacy of gene expression, and prevent the unintended spread of the virus, genetic modifications of adenoviruses have included the deletion of the E1 region, deletion of the E1 region along with deletion of either the E2 or E4 region, or deletion of the entire adenovirus genome except the cis-acting
10 inverted terminal repeats and a packaging signal (Gardlik et al., *Med Sci Monit.* 11: RA110-121, 2005).

[0043] Adeno-associated virus (AAV) vectors can achieve latent infection of a broad range of cell types, exhibiting the desired characteristic of persistent expression of a therapeutic gene in a patient. The discovery includes the use of any appropriate type of
15 adeno-associated virus known in the art including, but not limited to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6 and AAV7 (Lee et al., *Biochem J.* 387: 1-15, 2005). Previous experiments have shown that genetic modification of the AAV capsid protein can be achieved to direct infection towards a particular tissue type (Lieber, *Nature Biotechnology.* 21: 1011-1013, 2003). Modified serotype-2 and -8 AAV vectors in which tyrosine residues
20 in the viral envelope have been substituted for alanine residues that cannot be phosphorylated are also contemplated. In the case of tyrosine mutant serotype-2, tyrosine 444 is substituted with alanine (t2 mut 444). In the case of serotype 8, tyrosine 733 is substituted with an alanine residue (t8 mut 733). The titer for t2 mut 444 is 4.89E+12 and that for t8 mut 733 is 7.50E+13.

[0044] AAV vectors include those with a mutation of one or more surface-exposed tyrosine residues on capsid proteins. These mutated vectors avoid degradation by the proteasome, and significantly increase the transduction efficiency of these vectors. Mutation of one or more of the tyrosine residues on the outer surface of the capsid proteins including, for example, but not limited to, mutation of Tyr252 to Phe272 (Y252F), Tyr272 to Phe272
30 (Y272F), Tyr444 to Phe444 (Y444F), Tyr500 to Phe500 (Y500F), Tyr700 to Phe700 (Y700F), Tyr704 to Phe704), Tyr730 to Phe730 (Y730F) and Tyr733 to Phe733 (Y733F) provides improved transduction efficiency of the AAV vectors when compared to wild-type.

[0045] The modified vectors may facilitate penetration of the vector across the round window membranes, which would allow for non-invasive delivery of the vectors to the hair cells/spiral ganglion neurons of the cochlea. The EGFR-PTK (epidermal growth factor receptor--protein tyrosine kinase) phosphorylates tyrosine residues on the surface of the capsid targeting them for ubiquitinylation and degradation by the proteosome (Zhong, L, Zhao, W, Wu, J, Li, B, Zolotukhin, S, Govindasamy, L et al. (2007) A dual role of EGFR protein tyrosine kinase signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthesis. Mol Ther 15: 1323-1330). Using t2 mut 444 or t8 mut 733 it is possible to increase gene transfer by up to 10,000 fold decreasing the amount of AAV necessary to infect the sensory hair cells of the cochlea.

[0046] Using ex vivo gene therapy, an individual skilled in the art can be assured that the ESPN1, ESPN2 and/or ESPN3 (and optionally, ATOH1) protein or proteins will only be expressed in the desired tissue. In these applications, as well as applications where tissue specific expression of ESPN1, ESPN2 and/or ESPN3 (and optionally, ATOH1) is not a concern, the above vectors can be constructed to constitutively express ESPN1, ESPN2 and/or ESPN3 (and optionally, ATOH1) protein. Numerous constitutive regulator elements are well known in the art. Often, elements present in the native viruses described above are used to constitutively express a gene of interest. Other examples of constitutive regulatory elements include without limitation the chicken β -actin, EF1, EGR1, eIF4A1, FerH, FerL, GAPDH, GRP78, GRP94, HSP70, beta-Kin, ROSA, and ubiquitin B promoters.

[0047] For in vivo applications of gene therapy, the above vectors may be modified to include regulatory elements that confine the expression of ESPN1, ESPN2 and/or ESPN3 (and optionally, ATOH1) to certain tissue types. Numerous examples of regulatory elements specific to certain tissue types are well known in the art. Of particular interest to the discovery are elements that direct gene expression in the hair cells of the cochlea. An expression system for the inducible expression of ATOH1, that can find use for the inducible expression of ESPN1, ESPN2 and/or ESPN3 (and optionally, ATOH1) is described by Parker, *et al.*, *Hum Gene Ther Methods* (2014) 25(1):1-13.

[0048] In some examples, it may be desirable to direct ESPN1, ESPN2 and/or ESPN3 (and ATOH1) expression in an inducible fashion. Several methods of inducible transgene expression are widely used. These methods consist of the transfection of the patient's cells with multiple viral or plasmid vectors. Typically, a first vector expresses the gene of interest under the control of a regulatory element that is responsive to the expression

product of a second vector. The activity of this expression product is controlled by the addition of a pharmacological compound or some other exogenous stimulation. Examples of these systems are those that respond to tetracycline, mifepristone, ponasterone A, papamycin, tamoxifen, radiation, and heat shock (Robson et al., J. Biomed. Biotechnol. 2: 110-137, 2003).

[0049] Cochlear gene transfection in animals has utilized several approaches for vector delivery: (1) direct injection through round window membrane (RWM) into the perilymph, (2) intracochlear infusion through cochleostomy, and (3) transfusion through an intact RWM (Aarnisalo, *et al.*, ORL J Otorhinolaryngol Relat Spec. (2006) 68(4):220-7).

The third approach (transfusion through intact RWM) is least invasive and most likely to be accepted in human application.

[0050] The intact RWM consists of three layers: two epithelia layers separated by a layer of connective tissue, with collagen being a major component of the RWM. We have now demonstrated that the permeability of RWM can be increased temporarily by digestion of the membrane with collagenase. We then investigated whether (1) the digestion of RWM with the enzyme could facilitate the gene transfection of inner ear cells, and (2) if the digestion was safe to inner ear function and the structure of RWM. Instead of or in addition to enzymatic digestion of the RWM, proteidic delivery of polynucleotides across the RWM is described, *e.g.*, by Qi, *et al.*, *Gene Ther.* (2014) 21(1):10-8.

[0051] Additional approaches for delivery of a polynucleotide or polypeptide to the inner ear that can find use for delivering and ESPN polynucleotide or polypeptide include surgery facilitating viral delivery (Akil, *et al.*, *J Vis Exp.* (2015) Mar 16;(97)); nanoparticles and transtympanic injection (Zou, *et al.*, *J Nanobiotechnology.* (2015) 13:5); dendrimer-based nanocarriers (Wu, *et al.*, *J Biomed Nanotechnol.* (2013) 9(10):1736-45); and hyaluronic acid to enhance gene delivery (Shibata, *et al.*, *Hum Gene Ther.* (2012) 23(3):302-10).

4. Formulation, Administration, Dosing and Scheduling

a. Formulation and Administration of ESPN Polynucleotides

[0052] ESPN polynucleotides, *e.g.*, in a viral or plasmid vector, can be incorporated into pharmaceutical compositions suitable for administration to a subject. In some particular embodiments, the pharmaceutical composition comprises the vectors described herein and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier"

includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it can be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the vector or pharmaceutical composition.

[0053] The compositions described herein may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The form used depends on the intended mode of administration and therapeutic application. Typical compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans. The typical mode of administration is intratympanic (in the middle ear), intracochlear, parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular, intrathecal). In one example, the vector is administered by intravenous infusion or injection. In another example, the vector is administered by intramuscular or subcutaneous injection. In another example, the vector is administered perorally. In yet another example, the vector is delivered to a specific location using stereostatic delivery, particularly through the tympanic membrane or mastoid into the middle ear.

[0054] Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the vector in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

[0055] Generally, dispersions are prepared by incorporating the vector into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile lyophilized powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and spray-drying that yields a powder of the active ingredient plus any additional desired

ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be achieved by including
5 an agent in the composition that delays absorption, for example, monostearate salts and gelatin.

[0056] Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug
10 concentration. Sterile injectable solutions can be prepared by incorporating the vector in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

[0057] Generally, dispersions are prepared by incorporating the vector into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from
15 those enumerated above. In the case of sterile lyophilized powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and spray-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the
20 maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be achieved by including an agent in the composition that delays absorption, for example, monostearate salts and gelatin.

[0058] The vector used with some embodiments as described herein can be
25 incorporated into pharmaceutical compositions suitable for administration to a subject. In some particular embodiments, the pharmaceutical composition comprises the vectors described herein and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and
30 the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it can be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or

sodium chloride in the composition. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the vector or pharmaceutical composition.

5 **[0059]** The compositions described herein may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The form used depends on the intended mode of administration and therapeutic application. Typical compositions are in the form of
10 injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans. The typical mode of administration is intratympanic (in the middle ear), intracochlear, parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular, intrathecal). In one example, the vector is administered by intravenous infusion or injection. In another example, the vector is administered by intramuscular or
15 subcutaneous injection. In another example, the vector is administered perorally. In yet another example, the vector is delivered to a specific location using stereostatic delivery, particularly through the tympanic membrane or mastoid into the middle ear.

[0060] The vectors described herein can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of
20 administration will vary depending upon the desired results. In certain embodiments, the vector may be prepared with a carrier that will protect the vector against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters,
25 and polylactic acid. Many methods for the preparation of such formulations are generally known to those skilled in the art.

b. Formulation and Administration of ESPN Polypeptides

[0061] Compositions comprising ESPN polypeptides (and optionally an ATOH1 polypeptide) for otic administration (e.g., administration into the ear) will commonly
30 comprise a solution of the peptide comprising the peptide dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known

sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like.

5 The concentration of the one or more peptides in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[0062] Liquid form pharmaceutical preparations can include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. Aqueous solutions
10 suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizers, and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.
15 In varying embodiments, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

[0063] In various embodiments, the therapeutic agent is encapsulated in a liposome coated with or conjugated to one or more ESPN polypeptides. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions,
20 lamellar layers and the like. In these preparations the composition of the invention to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a desired target, such as antibody, or with other therapeutic or immunogenic compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol,
25 such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka, et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4,235,871; 4,501,728; 4,837,028 and 5,019,369. ESPN polypeptides can be integrated into, attached or conjugated directly to
30 the liposome using methods known in the art.

[0064] In some embodiments, the ESPN polypeptides are attached or conjugated to a liposome or a nanoparticle that encapsulates the therapeutic agent. Nanoparticles for encapsulation and delivery of a therapeutic agent are known in the art and can find use.

Illustrative nanoparticles include without limitation, e.g., semiconductor quantum dots (QDs), silicon (Si) nanoparticles (Park, et al., *Nature Materials* (2009) 8:331-336; Tu, et al., *JACS*, (2010) 132:2016-2023; Zhang, et al., *JACS*, (2007) 129:10668; Singh MP et al., *ACS Nano*, (2012) In press, (DOI: 10.1021/nn301536n); Rosso-Vasic, et al., *J. Mater. Chem.* (2009) 19:5926-5933; Bhattacharjee S., et al., *Nanotoxicology*, (2011) DOI 10.3109/17435390.2011.633714, 1 14; Chandra, et al., *Nanoscale* (2011) 3:1533-1540), polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents (Dinarvand, et al., *Int J Nanomedicine*. 2011;6:877-95); polyethyleneimine (PEI)-As(2)O(3)/Mn(0.5)Zn(0.5)Fe(2)O(4) magnetic nanoliposomes (Wang, et al., *Int J Nanomedicine*. 2011;6:871-5); redox-responsive poly(ethylene glycol)-b-poly(lactic acid) (MPEG-SS-PLA) nanoparticles (Song, et al., *Colloids Surf B Biointerfaces*. 2011, PMID 21719259); Thiolated Pluronic (Plu-SH) nanoparticles (Abdullah-Al-Nahain, et al., *Macromol Biosci*. 2011, PMID 21717576); and mesoporous silica nanoparticles (MSNs) (Wu, et al., *Chem Commun (Camb)*. 2011, PMID 21716992). In one embodiment, the ESPN polypeptides are conjugated to biocompatible nanomicelles comprised of cholic acid, lysine and polyethylene glycol (PEG) covalently conjugated together, e.g., described in Xiao, et al., *Biomaterials* (2009) 30:6006-6016; and Luo, et al., *Bioconjug Chem* (2010) 21:1216-1224. Further nanomicelles that find use are described, e.g., in PCT Patent Publ. WO 2010/039496.

[0065] In one embodiment, ESPN polypeptides are attached or conjugated to biocompatible nanomicelles comprised of cholic acid, lysine and polyethylene glycol (PEG) covalently conjugated together, e.g., as described in Xiao, et al., *Biomaterials* (2009) 30:6006-6016; and Luo, et al., *Bioconjug Chem* (2010) 21:1216-1224. Recently, a biocompatible nanomicelle drug delivery system comprised of a unique amphiphilic polymers called telodendrimers was developed [Xiao, et al., *Biomaterials* (2009) 30:6006-6016; Luo, et al., *Bioconjug Chem* (2010) 21:1216-1224]. Telodendrimers consist of cholic acid, lysine and polyethylene glycol (PEG) covalently conjugated together, which impart the ability to self-assemble into a water-soluble spheroid with a hydrophobic core capable of sequestering many types of drugs. Cholic acid, a primary component of bile acid, possesses a facial amphiphilic structure: a rigid steroid scaffold with four hydrophilic groups on one surface, and hydrophobic methyl groups on the other surface of the scaffold. Lysine is a natural amino acid. PEG is biocompatible and has been used to improve the pharmacokinetics of therapeutic drugs. This nanocarrier system has many attractive

characteristics for drug delivery, such as high drug loading capacity, narrow polydispersity, well-defined structure, easy chemical modification, superior physical, chemical stability and biocompatibility.

[0066] In some embodiments, the ESPN polypeptides in conjunction with a

therapeutic agent is formulated as a nanoparticle. Nanoparticle conjugates are known in the art and described, e.g., in Musacchio, et al., *Front Biosci.* (2011) 16:1388-412; Cuong, et al., *Curr Cancer Drug Targets.* (2011) 11(2):147-55; Jain, *BMC Med.* (2010) 8:83; Sunderland, et al., *Drug Development Research* (2006) 67(1):70-93; Gu, et al., *Nanotoday* (2007) 2(3):14-21; Alexis, et al., *ChemMedChem.* (2008) 3(12):1839-43; Fay, et al., *Immunotherapy.* (2011) 3(3):381-394; Minko, et al., *Methods Mol Biol.* (2010) 624:281-94; and PCT Publ. Nos. WO 2011/046842; WO 2010/040062; WO 2010/047765; and WO 2010/120385, the disclosures of which are hereby incorporated herein by reference in their entirety for all purposes. Known nanoparticle cores find use in encapsulating a therapeutic agent (e.g., an ESPN polynucleotide or polypeptide) for delivering to a tissue of interest, e.g., an inner ear auditory hair cell. One or more ESPN polypeptides can be integrated into, attached or conjugated directly to the nanoparticle core using methods known in the art. In some embodiments, the encapsulating nanoparticle is a cylindrical PRINT nanoparticle, e.g., as described in Gratton, et al., *Proc Natl Acad Sci USA.* (2008) 105(33):11613-8. The nanoparticle can be biodegradable or non-biodegradable, as appropriate or desired.

Poly(lactic acid-co-glycolic acid) (PLGA), biodegradable poly(L lactic acid) (PLLA) and PEG-based hydrogels find use as a matrix material in particle drug delivery systems because they are biocompatible, bioabsorbable, and have already shown promise in medical applications.

[0067] Peptide nanoparticles and methods for their preparation are known in the art

and described, e.g., in U.S. Patent Publication No. 2006/0251726, U.S. Patent Publication No. 2004/0126900, U.S. Patent Publication No. 2005/0112089, U.S. Patent Publication No. 2010/0172943, U.S. Patent Publication No. 2010/0055189, U.S. Patent Publication No. 2009/0306335, U.S. Patent Publication No. 2009/0156480, and U.S. Patent Publication No. 2008/0213377, each of which is hereby incorporated herein by reference in its entirety for all purposes. Further nanoparticle formulations that find use are described, e.g., in Emerich and Thanos, *Curr Opin Mol Ther* (2008) 10(2):132-9; Kogan, et al., *Nanomedicine* (2007) 2(3):287-306; Zhang, et al., *Bioconjug Chem* (2008) 19(1):145-152; Scarberry, et al., *J Am Chem Soc* (2008) 130(31):10258-10262; Fraysse-Ailhas, et al., *Eur*

Cells Materials (2007) 14(Suppl. 3):115; Corrias F, Lai F., Recent Pat Drug Deliv Formul. 2011 Aug 12, PMID:21834772; Wang, et al., Biomaterials. (2011) 32(32):8281-90; and Kaur, et al., Artif Cells Blood Substit Immobil Biotechnol. 2011 Aug 2., PMID:21806501. As appropriate, amino acid sequences may be added to either or both the N-terminus and the C-terminus of the peptide ligands in order to allow assembly and formation of the peptide nanoparticle.

c. Dosing

[0068] The pharmaceutical formulation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted predetermined fluid volumes in vials or ampoules.

[0069] The term "unit dosage form", as used in the specification, refers to physically discrete units suitable as unitary dosages for human subjects and animals, each unit containing a predetermined quantity of active material calculated to produce the desired pharmaceutical effect in association with the required pharmaceutical diluent, carrier or vehicle. The specifications for the novel unit dosage forms of this invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular effect to be achieved and (b) the limitations inherent in the art of compounding such an active material for use in humans and animals, as disclosed in detail in this specification, these being features of the present invention.

[0070] The pharmaceutical compositions described herein can include a "therapeutically effective amount" or a "prophylactically effective amount" of the vectors described herein. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, in this case for both prophylaxis and treatment of hearing loss or impairment of balance without unacceptable toxicity or undesirable side effects.

[0071] A therapeutically effective amount of the vector can vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the vector to elicit a desired response in the individual. A therapeutically effective amount can also be one in which any toxic or detrimental effects of the vector are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount

effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose can be used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount can be less than the therapeutically effective amount.

5 **[0072]** Dosage regimens can be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic response). For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It can be especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used
10 herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of vector calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms can be dictated by and directly
15 dependent on (a) the unique characteristics of the vector and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of formulating such vector for treating or preventing hearing loss or impaired balance in a subject.

[0073] Optimum dosages, toxicity, and therapeutic efficacy of compositions can further vary depending on the relative potency of individual compositions and can be
20 determined by standard pharmaceutical procedures in cell cultures or experimental animals, for example, by determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio, LD₅₀/ED₅₀. Compositions that exhibit large therapeutic indices are preferred. While
25 compositions that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compositions to the site of affected tissue to minimize potential damage to normal cells and, thereby, reduce side effects.

[0074] The data obtained from, for example, animal studies (e.g., rodents and monkeys) can be used to formulate a dosage range for use in humans. The dosage of
30 conjugated ESPN polypeptide lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration. For any composition for use in the methods of the invention, the therapeutically effective dose can

be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography (HPLC).

[0075] Following successful treatment, it may be desirable to have the subject undergo maintenance therapy to prevent the recurrence of the disease or malignant condition treated.

d. Scheduling

[0076] Dosing schedules can be calculated from measurements of peptides in the body of a subject. In general, dosage is from 1 ng to 1,000 mg per kg of body weight and may be given once or more daily, semiweekly, weekly, biweekly, semimonthly, monthly, bimonthly or yearly, as needed or appropriate. Persons of ordinary skill in the art can easily determine optimum dosages, dosing methodologies and repetition rates. One of skill in the art will be able to determine optimal dosing for administration of a peptide or peptide composition of the present invention to a human being following established protocols known in the art and the disclosure herein.

[0077] Single or multiple administrations of the pharmaceutical formulations may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the peptides of this invention to effectively treat the patient. Preferably, the dosage is administered once but may be applied periodically until either a therapeutic result is achieved or until side effects warrant discontinuation of therapy. Generally, the dose is sufficient to treat or ameliorate symptoms or signs of disease without producing unacceptable toxicity to the patient.

[0078] The daily dose can be administered once per day or divided into subdoses and administered in multiple doses, e.g., twice, three times, or four times per day. However, as will be appreciated by a skilled artisan, compositions described herein may be administered in different amounts and at different times. The skilled artisan will also appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or malignant

condition, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or, preferably, can include a series of treatments.

5 **[0079]** Thus, a pharmaceutical formulation thereof for intratympanic (in the middle ear) or intracochlear or inner ear administration would be about 0.01 to 100 mg/kg per patient per day. Dosages from 0.1 up to about 1000 mg/kg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Actual methods for preparing
10 parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remington: The Science and Practice of Pharmacy, 21st Ed., 2006, Lippincott Williams & Wilkins.

[0080] To achieve the desired therapeutic effect, pharmaceutical formulations may be administered for multiple days at the therapeutically effective daily dose. Thus,
15 therapeutically effective administration of compositions to treat a disease or malignant condition described herein in a subject may require periodic (e.g., daily) administration that continues for a period ranging from three days to two weeks or longer. In varying embodiments, compositions can be administered for at least three consecutive days, often for at least five consecutive days, more often for at least ten, and sometimes for 20, 30, 40
20 or more consecutive days, or longer, as needed. While consecutive daily doses are a preferred route to achieve a therapeutically effective dose, a therapeutically beneficial effect can be achieved even if the compounds or compositions are not administered daily, so long as the administration is repeated frequently enough to maintain a therapeutically effective concentration of the composition in the subject. For example, one can administer a
25 composition every other day, every third day, or, if higher dose ranges are employed and tolerated by the subject, once a week.

5. Methods of Monitoring

[0081] A variety of methods can be employed in determining efficacy of therapeutic and prophylactic treatments with the ESPN polynucleotides and/or ESPN polypeptides
30 described herein. Generally, efficacy is the capacity to produce an effect without significant toxicity. Efficacy indicates that the therapy provides therapeutic or prophylactic effects for a given intervention (examples of interventions can include by are not limited to administration of a pharmaceutical formulation, employment of a medical device, or

employment of a surgical procedure). Efficacy can be measured by comparing treated to untreated individuals or by comparing the same individual before and after treatment. Efficacy of a treatment can be determined using a variety of methods, including pharmacological studies, diagnostic studies, predictive studies and prognostic studies.

5 **[0082]** The methods of the present invention provide for improving hearing and inner ear hair cell function and/or reducing hearing loss and inner ear hair cell loss/destruction in a patient suffering from or susceptible to various middle ear and/or inner ear disorders, as described herein. A variety of methods can be used to monitor both therapeutic treatment for symptomatic patients and prophylactic treatment for asymptomatic
10 patients.

[0083] Monitoring methods can entail determining a baseline value of a monitored parameter (e.g., general ability to hear, ability to hear high frequency sounds, hair cell structure and function) in a patient before administering a dosage of the ESPN polynucleotides and/or ESPN polypeptides, and comparing this with a value for the same
15 parameter after treatment, respectively.

[0084] With respect to therapies using the ESPN polynucleotides and/or ESPN polypeptides, a significant decrease (e.g., greater than the typical margin of experimental error in repeat measurements of the same sample, expressed as one standard deviation from the mean of such measurements) in value of the measured parameter (e.g., hearing, hair cell
20 function) signals a positive treatment outcome (e.g., that administration of ESPN polynucleotides and/or ESPN polypeptides has blocked or inhibited, or reduced progression of the disease condition sought to be treated). In some embodiments, treatment with the ESPN polynucleotides and/or ESPN polypeptides is considered to be efficacious if the measured parameter in the subject being treated is reduced by at least about 10%, for
25 example, by at least about 20%, 30%, 40% or 50%, or by completely eliminating the symptoms of the measured parameter, e.g., comparing before and after treatment in the subject.

[0085] In other methods, a patient who is not presently receiving treatment but has undergone a previous course of treatment is monitored for one or more parameters or
30 symptoms of middle ear or inner ear disease to determine whether a resumption of treatment is required. The measured value of the symptom or parameter in the patient can be compared with a value of the same symptom or parameter previously achieved in the patient after a previous course of treatment. A significant decrease in the symptom or parameter

relative to the previous measurement (e.g., greater than a typical margin of error in repeat measurements of the same sample) is an indication that treatment need not be resumed. Alternatively, the value measured in a patient can be compared with a control value (mean plus standard deviation) determined in a population of patients after undergoing a course of treatment. Alternatively, the measured value in a patient can be compared with a control value in populations of prophylactically treated patients who remain free of symptoms of disease, or populations of therapeutically treated patients who show amelioration of disease characteristics. In all of these cases, a significant increase the symptoms or measured parameters relative to the control level (e.g., more than a standard deviation) is an indicator that treatment should be resumed in a patient.

EXAMPLES

[0086] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

Hair Cell Stereociliary Bundle Regeneration By Espin Gene Transduction After Aminoglycoside Damage And Hair Cell Induction By Notch Inhibition

The following example is published in Taura, *et al.*, *Gene Ther.* (2016) 23(5):415-23, which is hereby incorporated herein by reference in its entirety for all purposes.

MATERIALS AND METHODS

[0087] *Animals.* CD-1 mice of both sexes were purchased from Japan SLC Inc., Hamamatsu, Japan and bred under standard husbandry conditions. They were housed under SPF conditions in rodent boxes on sawdust bedding. Pups were used at 2 days after birth. Experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine and complied with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

[0088] *Production of Ad-espina vectors.* Replication-deficient recombinant adenoviruses with deleted E1, E3, and E4 regions 18 were used. Expression plasmids containing cDNAs for the Espin1-EGFP conjugate (Espin1-EGFP) and the Espin4-EGFP conjugate were kindly provided by Prof. James Bartles of Northwestern University. Adenovirus Espin1-EGFP (Ad-E1) and adenovirus Espin4-EGFP (Ad-E4) were constructed

using the Adenovirus Expression Vector Kit Ver. 2 (Takara) according to the manufacturer's protocol. In brief, the Espin1 or Espin4 cDNAs were transferred to a cosmid shuttle vector (pAxCawtit, Takara) containing the human cytomegalovirus promoter and SV40 termination sequence, and amplified. Then they were linearized by restriction enzyme digest. The expression inserts were transferred to the pAdEasy-1 adenoviral vector³⁵ by electroporation. The adenoviruses vectors were amplified in human embryonic kidney cells (HEK-293, RIKEN Bioresource Center, Cell No. RCB1637) and purified with the Adeno-X Maxi purification Kit (Clontech). We employed the vectors at a concentration of 1×10^7 total particles of purified virus per milliliter (pfu/ml). Viral suspensions were kept at -80°C until thawed for use. Appropriate protein production by Ad.E1-EGFP and Ad.E4-EGFP was confirmed by Western blotting following transduction of fibroblasts.

[0089] *Dissection of vestibular maculae and tissue culture.* Postnatal mice were decapitated under deep anesthesia and their temporal bones were removed. Utricula maculae were dissected from the surrounding tissue in 0.01M phosphate-buffered saline, pH 7.4. The otoconial membranes were gently removed with a fine needle. Explants of utricular sensory epithelia were placed intact on type I collagen-coated cover glasses (Iwaki, Tokyo, Japan) and maintained in 24-well culture plates (Iwaki) in Dulbecco's modified Eagle's medium (Invitrogen, Eugene, Oregon, USA), supplemented with 6 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 1.5 g/l penicillin G (Wako Pure Chemicals), at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hrs.

[0090] In order to examine the toxicity of gentamicin (GM) to HC stereociliary bundles and HC bodies, we damaged explants with 1mM GM (Nakarai, Japan) for 48 hrs and then maintained the explants in culture without GM for 10 days. After fixation with 4% paraformaldehyde and histochemistry (see below), we counted stereociliary bundles and HC bodies. Bundles and HC bodies were measured using a 10 X 40 eyepiece reticule. Each square of the reticule was 100 μm on a side. Surviving hair bundles and HC bodies were counted in each of three randomly selected fields including both striolar and extrastriolar regions, and the values obtained were averaged. Means and SDs were then converted into percentages for illustration in the figure. Five utricles were examined in each condition ($n=5$), based on our prior experience with treatment variability.

[0091] *Effect of notch inhibition.* To induce HC-like cells, two days after GM damage, we cultured utricular explants with 20 μM DAPT (N-[N-[(3,5-

Difluorophenyl)acetyl]-L-alanyl]-L-phenylglycinetert-butyl : C₂₃H₂₆F₂N₂O₄, Abcam) for 2 days, followed by 5 days without DAPT. DAPT is a prototypical γ -secretase inhibitor. Since Notch signaling requires γ -secretase activity, DAPT is a potent inhibitor of this signaling pathway. As a control, we cultured GM-damaged utricles for 7 days without
5 DAPT. Explants were analyzed for myosin7A immunoreactivity both as whole mounts and as tissue sections

[0092] *Transduction of Ad-espins.* Utricular maculae were cultured in DMEM overnight and then damaged with GM for two days. On the third day, we transduced 5 explants each with Ad-E1 or Ad-E4 (1 X 10⁷ pfu/ml) overnight. Using the same conditions,
10 we transduced adenovirus GFP (Ad-GFP) as a control. Explants were then cultured in DMEM for 7 days. For Notch inhibition, DAPT (20 μ M) was applied for 2 days, in between GM damage and adenovirus transduction.

[0093] *Observation GFP expression using time-lapse microscopy.* Beginning 70 hrs after adenovirus transduction, we evaluated GFP expression using time-lapse microscopy
15 (BZ9000, Keyence, Japan). Each of the five samples was observed three times per hr (20 mins) from 24 hrs after transduction up to 70 hrs. This method was used to identify the time of maximal GFP-adenoviral expression.

[0094] *Histochemical analysis.* At the end of the culture period, explants were fixed for 15 min in 4% paraformaldehyde and permeabilized with 5% Triton X-100 in phosphate-
20 buffered saline (PBS) after 3X PBS wash. Explants were then exposed to a blocking solution containing 10% bovine serum albumin (BSA), and then incubated with anti-myosin7A rabbit polyclonal antibodies (25-6790; Proteus Bioscience Inc., Ramona, CA, USA; 1:500). Alexa-Fluor 568 goat anti-rabbit IgG (A-11011, Invitrogen, CA, USA; 1:100) was used as the secondary antibody. At the same time, specimens were incubated in
25 FITC-conjugated or Alexa-Fluor 633 phalloidin (1:100; Invitrogen) to label F-actin for 1 hr. Finally, the explants were incubated with DAPI for 15 min. Specimens were examined using a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). Cells with phalloidin-positive surface structures were counted in randomly selected areas and averaged in each sample for each condition, using an
30 evaluation reticule 100 μ m on each side. Results from 6-7 samples were evaluated, based on initial observations of variability.

[0095] *Observation of stereociliary bundles by scanning electron microscopy*

(SEM). After the elimination of most stereociliary bundles by GM exposure, the process of hair bundle re-emergence induced by espin transduction was assessed by SEM. The explants were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde at 4°C for 4 hrs.

5 After fixation, they were dehydrated, dried, and coated with a thin layer of platinum palladium. The specimens were examined with a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan). Structures which exhibited clear stereociliary characteristics were counted in standard, randomly selected areas and averaged for each condition (n=6-8 explants, based on the variability exhibited by explants). Each square of
10 the evaluation reticule employed was 30 µm on each side.

[0096] *Functional analysis with FM1-43FX.* To evaluate the physiological function

of surviving HCs, we exposed GM-treated, DAPT exposed and transfected explants to FM1-43 (3-[4-[2-[4-(dibutylamino)phenyl]ethenyl] pyridin-1-ium-1-yl]propyl-
15 triethylazanium dibromide: C₃₀H₄₉Br₂N₃) FX dye (5µM, Invitrogen). FM1-43 is a

lipophilic, fluorescent dye that passes very rapidly through the MET channels located on
HC stereociliary bundles. For this reason, HC fluorescence after brief exposure to FM1-43
is commonly used to verify the functional status of the cells. The explants were transferred
to culture media supplemented with 5 µM FM1-43 for 10 seconds. During FM1-43
incubation, we applied mechanical stimulation via a fluid stream from a pipette, as
20 described previously (36). After fixation with 4% PFA, the specimens were examined with
a TCS-SP2 laser-scanning confocal microscope. Three independent assays were performed
in each condition. FM1-43 positive cells were counted in randomly selected areas and
averaged for each condition (n=6-9, based on prior studies). Each square of the evaluation
reticule was 100 µm on each side.

25 **[0097]** *Statistical analysis.* Sample sizes were chosen based on our prior experience

with variability in GN-exposed macular explants, to detect substantial differences in the
measured variables. Samples were excluded from the experiments if they failed to attach to
the culture surface or were folded. Because all samples were essentially identical, no
randomization was performed. Analysis was unblinded. Statistical analysis was performed
30 by analysis of variance (ANOVA) followed by the least significant difference (LSD) post-
hoc test with Bonferroni correction for repeated measures and two-sided t-test (Stat View
5.0), after determination of normal distribution and comparable variances between samples.

Differences associated with P values of less than 0.05 were considered to be statistically significant. All data are presented as mean $\pm$ standard deviation (SD).

RESULTS

[0098] *Gentamicin damage to the HC stereociliary bundles and cell bodies.* We

5 evaluated the number of HC somata and stereociliary bundles after gentamicin (GM) treatment of utricular maculae in vitro (Figure 1). After GM treatment, stereociliary bundles were almost entirely lost from the macular epithelium (Figure 1D) and the number of HC bodies was dramatically decreased. However, after an initial steep decline in HCs, the number was stable out to 10 days. Thus, a subset of HC somata, absent their stereocilia, 10 remained even 10 days after GM damage (Figure 1C).

[0099] *Transduction of macular explants.* We examined the transduction of GM-treated macular explants by Adenovirus-GFP (Ad-GFP), Ad.E1-EGFP (Ad-E1) and Ad.E4-EGFP (Ad-E4) adenoviral vectors. The maculae were well transduced by each vector, and the efficiencies of the three transductions were similar. Both HCs and supporting cells were 15 transduced (Figure 2). The expression of GFP in Ad-GFP transduced cells was diffused throughout the cell bodies (Figure 2A). In contrast, filamentous patterns of GFP were observed in Ad-E1 and Ad-E4 transduced cells (Figure 2B, 2C). These expression patterns appeared to be related to the actin-binding characteristics of espin.

[0100] *The effect of DAPT treatment.* As noted above, after GM damage, the 20 number of HCs present in macular explants decreased dramatically (Figure 3A, 3A', 3B, 3B'). However, damaged explants treated with DAPT exhibited substantially more cells expressing the HC marker myosin7A, especially at the edges of the explants. In sectioned samples, these myosin7A-positive cells were observed to lie primarily in the supporting cell layer (Figure 3C, 3C'). The number of myosin7A-positive cells in GM damaged explants 25 and DAPT-treated explants were respectively: 25.4 ± 5.5 (SD) and 38.5 ± 8.64 (SD) / 10000 μm^2 ($P < .05$, t test). However, stereociliary bundles remained very sparse in the DAPT-treated explants.

[0101] *Time-lapse microscopy after adenoviral transduction.* Espin1-GFP expression after Ad-E1 transduction of GM-damaged, DAPT-treated explants was 30 examined by time-lapse microscopy. Twenty-four hrs after vector administration, almost no GFP-positive cells were observed (Figure 4A). However, by 31 hrs, GFP expression had increased. By 46-48 hrs, expression reached a visual maximum and extensive filamentous

GFP expression was observed (Figure 4B, 4C, arrows). GFP expression then declined, but remained apparent for an additional 24 hrs. GFP signals following transduction with the other 2 adenoviruses (Ad-GFP and Ad-E4) increased, peaked, and declined at a slightly earlier stage.

5 [0102] *Influence of espin gene transduction on stereociliary bundles.* Transduction of damaged and regenerated explants with Ad-GFP or Ad-E4 had no effect on the number of stereociliary bundles. However, on the surfaces of Ad-E1 transduced explants a large increase in the number of phalloidin-positive structures which resembled immature stereociliary bundles was noted (Figure 5A). In sectioned explants, the many myosin7A-
10 positive cells that were apparent in the supporting cell layer exhibited GFP-positive extensions that reached from the supporting cell layer to the apical surface of the epithelium. Moreover, phalloidin-labeled stereociliary bundle-like structures projected from the apical surfaces of these extensions (Figure 5B). While some stereociliary bundles were observed in Ad-GFP- or Ad-E4-transduced explants (11.4 ± 6.87 (SD) / $10000 \mu\text{m}^2$), a
15 substantially and significantly greater number were observed on Ad-E1-transduced macular explants (28.8 ± 7.82 (SD) / $10000 \mu\text{m}^2$; $P < .05$, t-test) (Figure 5C).

[0103] *Scanning electron microscopy (SEM).* After Ad-GFP transduction, the reticulated boundaries between cells were clearly apparent on the surfaces of explants imaged by SEM. Small microvilli and basal bodies were also observed on the surfaces of
20 the cells, but stereociliary bundles were essentially absent (Figure 6A). In contrast, on the surface of Ad-E1 transduced explants, many immature stereociliary bundle-like structures were observed (Figure 6B). However, there were very few immature stereociliary bundle-like structures in Ad-E4 transduced explants, although elongated microvillus-like elements were seen (Figure 6C). When the stereociliary bundle-like structures were quantified, the
25 number on Ad-E1 transduced explants was substantially and significantly ($p < .05$, t-test) greater than in Ad-GFP or Ad-E4 transduced epithelia (Figure 6D). Higher magnification images revealed recognizable bundles of stereocilia, with a typical stair-step arrangement, on Ad-E1 transduced explants. These bundles exhibited a single, central kinocilium, and stereocilia were present on approximately one half of the apical surface of each bundle-
30 bearing cell (Figure 7A). These stereocilia appeared to be linked at their tops (Figure 7B).

[0104] *Functional analysis of stereociliary bundles using FM1-43FX loading.* After brief treatment with FM1-43FX, only a small number of fluorescent cells were observed in Ad-GFP and Ad-E4 transduced explants (Figure 8A, 8C). However, substantially more

FM1-43FX-positive cells were observed in Ad-E1 transduced explants (Figure 8B). Quantitative analysis of FM1-43 positive cells in explants revealed dramatic and significant ($p < .05$) differences in labeling between Ad-E1 transduced explant and those transduced with either Ad-GFP or Ad-E4 (Figure 8D). These results provide strong evidence that stereociliary bundles on Ad-E1 transduced explants exhibit functional mechanoelectrical transduction channels.

DISCUSSION

[0105] Gene therapy has proven to be a promising tool for the correction of gene deficiencies and for introducing beneficial gene expression into a variety of tissues.

Recently, advances have been reported in the use of gene therapy for inner ear disorders (12). In this study, we evaluated the potential for gene therapy-induced *espin* overexpression to regenerate HC stereociliary bundles after HC damage and induction. When HCs were damaged with aminoglycosides, their apical surface including the hair bundle was rapidly lost, as has been previously reported (4). This was followed by the degeneration of the HC soma for most cells. However, a subpopulation of HCs survived, absent their stereocilia, even at 10 days post aminoglycoside treatment. Again, this has been reported in prior studies (19). Treatment of damaged cultures with the γ -secretase inhibitor DAPT resulted in the generation of additional HCs, but these cells lacked stereociliary bundles in our study. This agrees with the results of some prior evaluations of HC regeneration (17,19) although stereocilia have been reported on regenerated mammalian HCs in other studies, especially when longer periods of regeneration are involved (15, 18) Transduction with Espin1-EGFP in an adenoviral vector led to robust growth of stereociliary bundles, while transduction with an Espin4-EGFP or GFP alone was ineffective.

[0106] As noted above, espins are actin bundling proteins produced in multiple isoforms from a single gene. Espin mutant mice show abnormal stereocilia, indicating the importance of this gene for these structures (9). Moreover, specific *espin* isoforms have the potential to be differentially involved in stereociliogenesis during HC development. In the inner ear HCs of altricial rodents, the *espin1* isoform accumulates during the late embryonic and early postnatal periods, while the *espin2* and 3 isoforms predominate in the embryonic inner ear until approximately E20. Therefore, espins1-3 are candidates for involvement in stereociliogenesis, with *espin1* expression most closely matching the period of stereociliary development (9). In contrast, the *espin4* isoform accumulates between postnatal days 6 and

10, after stereocilia have reached their essentially adult characteristics (9). This suggests that *espin4* is less likely to be involved in stereociliogenesis. However, when *espin4* is over-expressed in other cell types, microvilli are elongated (23) and in cochlea both microvilli and stereocilia are similarly elongated (24). Therefore, more than one *espin* isoform has the capacity to influence stereocilia formation. We therefore tested the ability of *espin1* and *espin4* overexpression to generate stereocilia in damaged and regenerated HCs. In Ad-E1 transduced explants, we observed phalloidin-positive stereocilia bundle-like structures on myosin7A-positive cells. SEM study revealed that only in Ad-E1 transduced explants did we observe recognizable stereocilia with a staircase pattern. This, in combination with the data cited above, suggests that *espin4* may be more involved in the elongation than in the genesis of stereocilia. However, it should of course be noted that the fusion of *espin4* to GFP might have altered its properties in our experiments. Any conclusions regarding the lack of effect of *espin4* transduction on stereociliary bundle formation should keep this in mind.

[0107] These results suggest that *espin1* gene therapy might be effective as a means of regenerating stereociliary bundles. However, it does not necessarily follow that the stereocilia are functional. Rapid FM1-43 accumulation in HCs, on the order of seconds, is thought to be mediated by entry through functional mechanoelectrical transduction channels (25,26). Although endocytotic entry has been reported in guinea pig inner HCs, this occurs with substantially slower kinetics (27). Therefore, rapid FM1-43 loading is thought to reflect the physiological function of HC mechanotransduction channels at the tips of the stereocilia (26). In Ad-E1 transduced explants, the number of FM1-43 positive cells observed after brief exposure was substantially and significantly ($p < .05$, t-test) larger than the other two experimental groups. This suggests that stereocilia induced by *espin1* gene therapy bear functional MET (mechanoelectrical transduction) channels.

[0108] The fact that *espin1* overexpression alone increased the formation of stereociliary arrays is perhaps surprising, given that actin-crosslinking presumably represents only part of the process required for stereociliary formation. This suggests that the genetic program for stereociliogenesis is partially active in damaged and/or regenerated HCs, but lacks *espn* gene expression as a critical component. The reason for this lack is unclear. It can be assumed that *espin* expression during development is initiated by the activity of specific HC fate and differentiation genes. Transduction of nonsensory inner ear cells with the transcription factor ATOH1 induces the formation of HC-like cells, some of

which will form stereocilia, although they are often immature (13,18). ATOH1 overexpression has also been shown to induce stereociliogenesis in damaged HCs, although again some such stereocilia are immature (28). Therefore ATOH1 alone does not appear to be sufficient to specify appropriate espn gene expression. ATOH1 is known to directly regulate the gene encoding the HC differentiation transcription factor POU4F3 (29), and animals that lack the pou4f3 gene fail to develop stereocilia (30). It is thus possible that POU4F3 directly regulates the espn gene. We therefore evaluated the 1500 bp region 5' to the start site of the murine espn gene for consensus POU4F3 binding sites, as identified by Xiang et al., (31) or the JASPAR database. Two sites were found ~650-800 bp 5' to the espn transcription initiation site. This provides suggestive, but by no means sufficient, evidence of POU4F3 regulation. In any case, low levels of expression of POU4F3, or of other HC differentiation factors, may explain the insufficient espn transcription in damaged and regenerated HCs.

[0109] Differentiated HCs express Notch, and the Notch pathway inhibits adjacent supporting cells from differentiating into HCs (32-34). Lin et al. (19) reported that HC regeneration was promoted by Notch inhibition, with conversion of supporting cells to HCs and increased atoh1 transcriptional activity. This is consistent with our results in which many more Myosin7A-positive cells were observed in damaged epithelia that were treated with DAPT. In our study, we did not observe stereociliary bundles on the myosin7A-positive cells induced by Notch inhibition alone within 7days. This suggests that Notch signaling may not be sufficient for complete development of HCs, which might otherwise be expected if Notch effects are mediated by increased ATOH1. Alternatively, stereociliary regeneration induced by Notch inhibition may require additional time.

[0110] In conclusion, espin1 gene therapy appears be useful for significantly enhancing morphological and physiological stereociliary bundle regeneration on damaged and regenerated HCs. The combination of Notch inhibition and espin1 gene transduction is therefore a promising candidate for inner ear regenerative therapy in future. However, it must be noted that our experiments were performed in neonatal mice. Future studies will be needed to determine whether this regenerative effect occurs in adult mice, a necessary pre-requisite for potential clinical utility.

REFERENCES

1. Nadol Jr JB. Hearing loss. *N Engl J Med* 1993; 329: 1092–1102.
2. Roberson DF, Weisleder P, Bohrer PS, Rubel EW. Ongoing production of sensory cells in the vestibular epithelium of the chick. *Hear Res* 1992; 57: 166-174.
- 5 3. Ryals BM, Rubel EW. Hair cell regeneration after acoustic trauma in adult *Coturnix* quail. *Science*. 1988; 240: 1774-1776.
4. Gale JE, Meyers JR, Periasamy A, Corwin JT. Survival of bundleless hair cells and subsequent bundle replacement in the bullfrog's saccule. *J Neurobiol*. 2002; 50: 81-92.
5. Jia S, Yang S, Guo W, He DZ. Fate of mammalian cochlear hair cells and stereocilia
10 after loss of the stereocilia. *J Neurosci*. 2009; 29: 15277-15285.
6. Sekerková G, Zheng L, Loomis PA, Mugnaini E, Bartles JR. Espins and the actin cytoskeleton of hair cell stereocilia and sensory cell microvilli. *Cell Mol Life Sci*. 2006; 63: 2329-2341.
7. Sekerkov G, Zheng L, Mugnaini E, Bartles JR. Differential expression of espin isoforms
15 during epithelial morphogenesis, stereociliogenesis and postnatal maturation in the developing inner ear. *Dev. Biol*. 2006; 291: 83–95.
8. Li H, Liu H, Balt S, Mann S, Corrales CE, Heller S. Correlation of expression of the actin filament-bundling protein espin with stereociliary bundle formation in the developing inner ear. *J. Comp. Neurol*. 2004; 468: 125–134.
- 20 9. Rzadzinska A, Schneider M, Noben-Trauth K, Bartles JR, Kachar B. Balanced levels of Espin are critical for stereociliary growth and length maintenance. *Cell Motil Cytoskeleton*. 2005; 62: 157-165.
10. Zheng L, Sekerková G, Vranich K, Tilney LG, Mugnaini E, Bartles JR. The deaf jerker mouse has a mutation in the gene encoding the espin actin-bundling proteins of hair cell
25 stereocilia and lacks espins. *Cell*. 2000; 102: 377-385.
11. Van de Water TR, Staecker H, Halterman MW, Federoff HJ. Gene therapy in the inner ear. Mechanisms and clinical implications. *Ann N Y Acad Sci*. 1999; 884: 345-360.
12. Ryan AF, Dazert S. Gene therapy for the inner ear: challenges and promises. *Adv Otorhinolaryngol*. 2009; 66: 1-12

13. Kawamoto K, Yagi M, Stover T, Kanzaki S, Raphael Y. Hearing and hair cells are protected by adenoviral gene therapy with TGF-beta1 and GDNF. *Mol. Ther.* 2003; 7: 484-492.
14. Kawamoto K, Sha SH, Minoda R, Izumikawa M, Kuriyama H, Schacht J et al.
5 Antioxidant gene therapy can protect hearing and hair cells from ototoxicity. *Mol Ther* 2004; 9: 173–181.
15. Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF et al. Auditory hair cell replacement and hearing improvement by *Atoh1* gene therapy in deaf mammals. *Nat Med* 2005; 11: 271–276.
- 10 16. Schlecker C, Praetorius M, Brough DE, Presler RG Jr, Hsu C, Plinkert PK, et al. Selective atonal gene delivery improves balance function in a mouse model of vestibular disease. *Gene Ther.* 2011; 18: 884-890.
17. Tona Y, Hamaguchi K, Ishikawa M, Miyoshi T, Yamamoto N, Yamahara K, et al. Therapeutic potential of a gamma-secretase inhibitor for hearing restoration in a guinea pig
15 model with noise-induced hearing loss. *BMC Neurosci.* 2014; 15: 66.
18. Mizutani K, Fujioka M, Hosoya M, Bramhall N, Okano HJ, Okano H, et al. Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. *Neuron.* 2013; 77: 58-69.
19. Lin V, Golub JS, Nguyen TB, Hume CR, Oesterle EC, Stone JS. Inhibition of Notch
20 activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J Neurosci.* 2011; 31: 15329-39.
20. Ahmed M, Xu J, Xu PX. EYA1 and SIX1 drive the neuronal developmental program in cooperation with the SWI/SNF chromatin-remodeling complex and SOX2 in the mammalian inner ear. *Development.* 2012; 139: 1965-77.
- 25 21. Atkinson PJ, Huaraca Najarro E, Sayyid ZN, Cheng AG. Sensory hair cell development and regeneration: similarities and differences. *Development.* 2015; 142: 1561-1571.
22. Ryan AF. The cell cycle and the development and regeneration of hair cells. *Curr Topics Devel Biol* 2002; 57: 449-466.

23. Loomis PA, Zheng L, Sekerková G, Changyaleket B, Mugnaini E, Bartles JR. Espin cross-links cause the elongation of microvillus-type parallel actin bundles in vivo. *J Cell Biol.* 2003; 163: 1045-1055.
24. Rzadzinska A, Schneider M, Noben-Trauth K, Bartles JR, Kachar B. Balanced levels of
5 Espin are critical for stereociliary growth and length maintenance. *Cell Motil Cytoskeleton.* 2005; 62: 157-165.
25. Gale JE, Marcotti W, Kennedy HJ, Kros CJ, Richardson GP. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *J Neurosci.* 2001; 21: 7013-7025.
- 10 26. Meyers JR, MacDonald RB, Duggan A, Lenzi D, Standaert DG, Corwin JT, et al. Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. *J Neurosci.* 2003; 23: 4054-4065.
27. Griesinger CB, Richards CD, Ashmore JF. FM1-43 reveals membrane recycling in adult inner hair cells of the mammalian cochlea. *J Neurosci.* 2002; 22: 3939-3952.
- 15 28. Yang SM, Chen W, Guo WW, Jia S, Sun JH, Liu HZ, et al. Regeneration of stereocilia of hair cells by forced *Atoh1* expression in the adult mammalian cochlea. *PLoS One.* 2012; 7: e46355.
29. Masuda M, Dulon D, Pak K, Mullen LM, Li Y, Erkman L, et al. Regulation of *POU4F3* gene expression in hair cells by 5' DNA in mice. *Neuroscience.* 2011; 197: 48-64.
- 20 30. Erkman L, McEvelly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM, et al. Role of transcription factors *Brn-3.1* and *Brn-3.2* in auditory and visual system development. *Nature.* 1996; 381: 603-606, 1996.
31. Xiang M, Zhou L, Macke JP, Yoshioka T, Hendry SH, Eddy RL, et al. The *Brn-3* family of *POU*-domain factors: primary structure, binding specificity, and expression in
25 subsets of retinal ganglion cells and somatosensory neurons. *J Neurosci.* 1995; 15: 4762-4785.
32. Yamamoto N, Tanigaki K, Tsuji M, Yabe D, Ito J, Honjo T. Inhibition of Notch/RBP-J signaling induces hair cell formation in neonate mouse cochleas. *J Mol Med (Berl)* 2006; 84: 37-45.

33. Hori R, Nakagawa T, Sakamoto T, Matsuoka Y, Takebayashi S, Ito J. Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea. *Neuroreport* 2007; 18: 1911-1914.

34. Murata J, Ikeda K, Okano H. Notch signaling and the developing inner ear. *Adv Exp Med Biol.* 2012; 727: 161-173.

35. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A.* 1998; 95: 2509-2514.

36. Taura A, Kojima K, Ito J, Ohmori H. Recovery of hair cell function after damage induced by gentamicin in organ culture of rat vestibular maculae. *Brain Res.* 2006; 1098: 33-48.

[0111] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

INFORMAL SEQUENCE LISTING

Sequence ID No: 1 – human espin isoform 1 nucleic acid sequence (mRNA; NCBI Reference Sequence: NM_031475.2)

```

5           1 agcggagcgc caggcagcgc ggagcggagg ccaggcccac
agccgctccg cctcccggcc
           61 cgcagatccc cgacggccgc accgcggggt cctctggccc
gcaagaacac gtgcatggcg
           121 tcctggggaa ggcgctgagt gcggagtcgc ggcgccgcac
10  gcggcaccat ggccctggag
           181 caggcgctgc aggcggcgcg gcagggcgag ctggacgtgc
tgaggtcgct gcacgccgca
           241 ggcctcctgg ggccctcgct gcgcgaccgc ctggacgcgc
tgcccgtgca ccacgcggcc
15           301 cgcgctggga agctgcactg tctgcgcttc ctggtggagg
aagccgccct ccccgccgcg
           361 gcccgcgccc gcaacggcgc cacaccggcc cagcagcct
ccgccaccgg ccacctcgcc
           421 tgcctgcagt ggctgctgtc gcagggcggc tgcagagtgc
20  aggacaaaga caattctggt
           481 gccacagtct tgcattctggc tgcccgttcc ggccaccccg
aggtggtgaa ctggctcttg
           541 catcatggcg gtggggaccc caccgcggcc acagacatgg
gcgccctgcc tatccactac
25           601 gctgccgcca aaggagactt cccctccctg aggcttctcg
tcgagcacta ccctgagggg
           661 gtgaatgccc aaaccaagaa cggtgccacg cccctgtacc
tggcgtgcca ggagggccac
           721 ctggaggtga ccagctacct ggtgcaggaa tgcggcgcag
30  acccgcacgc gcgcgcccac
           781 gacggcatga ccccgctgca cgccgcggcg cagatggggc
acagcccagt catcgtgtgg
           841 ttggtgagct gcaccgacgt gagcctgtcc gagcaggaca
aagacggcgc caccgccatg
35           901 cacttcgcgg cgagccgcgg ccacaccaag gtgctcagct
ggctgctgct gcacggcggg
           961 gagatctcgg ctgacctgtg gggcgggacc ccgctgcacg
acgccgccga gaacggggag
           1021 ctagagtgct gccagatcct ggtagtgaac ggcgcggagc
40  tggacgtccg cgaccgcgac
           1081 gggtagacgg ccgccgacct gtcggacttc aacggccaca
gccactgcac ccgctacctg
           1141 cgcacgggtg agaacctgag cgtggagcac cgcgtgcttt
cccgggatcc atccgcagag
45           1201 ctggaggcta agcagccgga ttcaggcatg tcctcaccca
ataccacggg gtcggtccag
           1261 ccgctgaact ttgacctcag ctcgcctacc agcaccctct
ccaactacga ctctgctcc
           1321 tccagccact ccagcatcaa gggccagcac cctccatgtg
50  ggctttccag cgctagagct

```

1381 gcagacatac agagctacat ggacatgctg aaccgaggagc
 tgggcctgcc tcggggcacg
 1441 attgggaagc ccacaccccc accaccccca cccagcttcc
 ccccgccacc cccgccccca
 5 1501 ggcacccaac tgccccacc cccacctggc taccagctc
 ccaagcctcc tgtaggacca
 1561 caggcagctg acatctacat gcagaccaag aacaaactcc
 gccacgtgga gacagaggcc
 1621 ctcaagaagg agctgagctc ctgtgacggc cacgacgggc
 10 tgcggaggca ggactccagc
 1681 cgcaagcccc gcgccttcag caagcagccc agcacggggg
 actactaccg gcagctgggc
 1741 cgctgccccg gcgagacgct ggccgcacgc ccgggcatgg
 cgcacagcga ggaggtgcgt
 15 1801 gcccggcagc ccgcgcgcgc cggctgcccg cgcctcggcc
 ctgccgcccg cggctcactc
 1861 gaaggcccct ccgctccccc gcaggcggcg ctgcttctctg
 ggaacctatgt tcctaacggc
 1921 tgcgccgcgg accccaaggc gtccaggag ctgccaccgc
 20 cgcgccacc gccgcgcgcg
 1981 cccctgccgg aggcgcgcgag ttcgccaccg ccggccccgc
 ctctgcccct cgagagcgct
 2041 ggccctggct gcgggcagcg ccgctcctcc tcgtccaccg
 gcagcaccaa gtctttcaac
 25 2101 atgatgtccc cgacggggcga caactcggag ctactggctg
 agattaaggc aggcaagagc
 2161 ctgaagccga cgccccagag caaggggctg accacagtgt
 tctcaggcat cgggcagccg
 2221 gccttccagc ccgattcgcc gctgccttct gtgtcacctg
 30 cactgtcacc agtccggagc
 2281 cccacaccgc cagctgcggg gtttcagccg ctgctcaatg
 gaagcttggc tcccgtgccg
 2341 cccactactc ctgcgcggg agtgcagctg gacgtggagg
 ctctcatccc cacgcacgat
 35 2401 gagcagggcc ggcccatccc cgagtggag cgccagggtga
 tgggtgcgca gatgcagctg
 2461 aagatgcagg aggaggagga gcagaggcgg aaggaggagg
 aggaggaggc ccgctggcc
 2521 agcatgccc cctggaggcg ggacctctg cggaagaagc
 40 tggagaaga gagggagcag
 2581 aagcggaag aggaggagcg acagaagcag gaggagctgc
 ggcgggagaa ggaacagtca
 2641 gagaagctgc ggacgctggg ctacgatgag agcaagctgg
 cgccctggca gcgacaggtc
 45 2701 atcctgaaga agggggacat cgctaagtac tagaggccgc
 agactcctgt ccgcagcctc
 2761 gcagctccgt ggggccctcc gcccagccc cagccagcca
 ggccctgggtg gaaaggctgg
 2821 gagccgcaca gccctcccct cctgcgctgg aaaccctccc
 50 tgacccccac cctggcccc

2881 cgtatcccca gcccttggca acactggagt gcacacgccg
 ccacggttgc ccagaaaaag
 2941 tgcccaagct gctgacgcaa acaacaacaa atgctgctta
 tttgcatgcc gacttacata
 5 3001 tatttgcattg ttcgttgact atcaaagagt gcagagctct
 cccagagccc gtgggtggtg
 3061 actttgtttt cctgcggggc tcagccccct ccaggatgca
 gccccctccc ccgcacccccg
 3121 gaaccggcgt cgctggcgca tcctgggtgg aggcaggccc
 10 cgagctcggg gaaggggttt
 3181 tcccttcctc tctgaccag atctgcgcgc ggcctagccc
 gggcctcatt tcttatcccc
 3241 gccaaagggt tcctctcagt catttggtta ccagaaacat
 gaaaactgcc tgtctggccg
 15 3301 ggccgcactt gtggcccccg ggaccccacc tctggcccca
 cctccctcaa gtctgcgccc
 3361 cgtccccagc cagaccact cgctgccggg accctttcac
 tgccccggtg gagtgaatag
 3421 aggatgaggg gccctgacc tgtgtctcca actgctgcac
 20 cccatcccga ccctgtctcc
 3481 gccacctcgc agccccatta aagcgctctc atctgggctc
 cggttcactc a

25 **Sequence ID No: 2 - human espin isoform 1 amino acid sequence**
(NCBI Reference Sequence: NP_113663.2)

1 malegalqaa rggeldvlrs lhaagllgps lrdpldalpv
 hhaaragklh clrflveeaa
 61 lpaaararng atpahdasat ghlaclqwl sgggcrvqdk
 30 dnsgatvlhl aarfghpevv
 121 nwlhgggd ptaatdmgal pihyaaakgd fpslrlveh
 ypegvnaqtk ngatplylac
 181 qeghlevtqy lvqecgadph arahdgmtp lhaaaqmghsp
 vivwlvscd vslseqdkdg
 35 241 atamhfaasr ghtkvlswll lhggeisadl wggtplhdaa
 engeleccqi lvnngaeldv
 301 rdrdgytaad lsdfngshc trylrvtenl svehrvlsrd
 psaeleakqp dsgmsspntt
 361 vsvqplnfdl ssptstlsny dscssshssi kgqhppcgl
 40 saraadiqsy mdmlnpelgl
 421 prgtigktp ppppsfppp ppppgtqlpp pppgypapkp
 pvgpqaadiy mqtknklrhv
 481 etealkkels scdghdglrr qdssrkpraf skqpstgdy
 rqlgrcpget laarpgmahs
 45 541 eevrarqpar agcprlgpaa rgslegpsap pqaallpgnh
 vpngcaadpk asrelppppp
 601 pppplpeaa sspppaplp lesagpgcgq rrsssstgst
 ksfnmmsptg dnsellaek
 661 agkslkptpq skglttvfsg igqpafqps plpsvspals
 50 pvrspptpaa gfpplngsl

721 vpvppttpap gvqldveali pthdeqgrpi pewkrqvmvr
 kmqlkmqeeee eqrrkeeeee
 781 arlasmpawr rdllrkklee ereqkrkeee rqkqeelrre
 keqseklrtl gydesklapw
 5 841 qrqvilkkgd iaky

Sequence ID No: 3 – Mus musculus espin (Espn), transcript variant 1, mRNA (NCBI Reference Sequence: NM_207687.2)

10 1 atggccctgg agcaggcgct gcaggcggca cggcggggcg
 acctggacgt gctgaggtcc
 61 ctgcacgccg ccggcctgct ggggccttct ctgcgcgact
 cgctagacgc cctgccggtg
 121 caccatgcgg cccgctcagg caagctgcac tgtctgcgct
 15 acttggtgga ggaggttgcc
 181 ctcccagccg tgtcccgcgc gcgcaacggc gccacaccag
 cccatgacgc cgccgccaca
 241 ggctacctct cttgcctgca gtggctgctc acacaggggtg
 gctgcagggg gcaggaaaaa
 20 301 gataactctg gtgccacagt cctgcatctg gctgcccgt
 ttggccaccc ggatgtggtg
 361 aagtggctgc tgtaccaggg cggtgcaaat tcggccatca
 ccacagacac gggcgccctg
 421 cctatccact acgctgccgc caaaggagac ctcccctcgc
 25 tgaagcttct ggtcgggcat
 481 taccctgagg gagtgaatgc ccaaaccaac aacggtgcca
 cgcccctgta cctggcgtgc
 541 caggagggcc acctggaagt gacgaagtac cttgtgcagg
 agtgcagtgc agatccgcac
 30 601 ctgcgcgccc aagacggcat gacacccta catgccgcag
 cacagatggg ccacaaccca
 661 gtcttggtgt ggctggtgag ctttgcagac gttagctttt
 cagagcagga ccacgacggc
 721 gccacggcca tgcactttgc tgccagtcgc ggccacacca
 35 aagtgtcag ctggctcctg
 781 ctgcacgggg cagagatctc gcaggacctg tggggcgga
 ccccgctgca tgacgctgct
 841 gagaacgggg aactggagtg ctgccagatc ctgcggtga
 acggcgctgg gctggacgtc
 40 901 cgcgaccacg atgggtacac cgctgccgac ctggcagagt
 tcaatggcca caccactgt
 961 tcccgctacc tacgtacggg gcaaaccctg agcttggaac
 accgagtctt gtcccgggat
 1021 caatccatgg acctggaggc aaagcagctg gactcgggta
 45 tgtcctcgcc caacaccacc
 1081 atgtcgggtcc agccaatgac ctttgacctg ggctcgccta
 ctagcacgtt ctccaactat
 1141 gactcctgct cctccagtca ctccagcagc aaggggcagc
 gatcgaatcg agggattcca
 50 1201 ggtgcaagag ctgcagactt acagagctac atggacatgc
 tgaaccacaga gaagagcttg

1261 cctcggggca agctagggaa gccttccccg ccaccacctc
 caccaccacc accaccaagc
 1321 ttcccgccac cccaccacc cacaggcacc cagccgcccc
 cacctccacc aggctaccca
 5 1381 gctcccaatc cccctgtggg actgcatctg aataacattt
 acatgcagac caagaacaag
 1441 cttcgccatg tggaggtgga ctcgctcaag gagcccaagg
 tggagctgaa cgatcagttt
 1501 gcacagccga gctcggggcga cggccactcg gggctacaca
 10 ggcaggactc cgggctgctc
 1561 aggcaggatt cggagctgct gcacaggcag gagctgctca
 ggcacagcac cggactgcgc
 1621 aggcaggact ccgaccgcaa acagcgctcg ttcagtaaac
 agcccagcac gggggactac
 15 1681 taccgccagc tgggccgcag cccgggggag ccgctggccg
 cagccccggg catggccac
 1741 agcgaggagg cggcgctgct ccccggaac cacgtgcaca
 acggctgctc agcggactcc
 1801 aaagcgtcca gggagctgcc gccgccaccg ccgccgccgc
 20 cgctgccga ggccctgagt
 1861 tcgccgccgc ccgcccacc tctgcccatc gagggcgcg
 gcgcagcctg cgggcagcgt
 1921 cgttcctcgt cttctactgg caaagtgaga gtcctgagac
 acaggaagag caccaaattc
 25 1981 ttcaacatga tgtccccaac gggtgataac tcagagcttc
 tggctgagat aaaggcgggc
 2041 aagagcctga agccgacacc gcagagcaag gggctgacaa
 ccgtgttctc aggcagtggg
 2101 cagccagcct ccagcctga gtcaccgcag cctctgggtgt
 30 cacctgcgcc atctcggact
 2161 cggagcccca cccgccagc ctctgggtct cagccactgc
 tcaatggcag tgtggtgccg
 2221 gcaccacctg ccaccccggc acctggagtc catctggatg
 tggaggccct cattcccact
 35 2281 cttgatgagc agggccggcc catcccggag tggaaagcgc
 aggtgatggt ccgcaagctg
 2341 cagcagaaga tgcaggagga agaggagcag cggagggaagg
 aggaagagga ggaggcccg
 2401 ctgccagcc tgctgcctg gagacgagac attcttcgga
 40 agaagctgga ggaggagagg
 2461 gagcagaagc gaaaagagga ggagcggcaa aagctggagg
 aaatacagag ggcgaaagaa
 2521 cagtcggaga agctgcggac actaggctac gacgaagcca
 agctcgcgcc ctggcagcga
 45 2581 caggatcatc tgaagaaggg ggagatccct aagtaatagg
 agtctctcgg cttcttgctg
 2641 gtagcctcac aagttctgag agatggggag cggccccag
 cccccgccc atcctgtcag
 2701 gttgggtgcg aagggttagg atccttgccg cccttccctt
 50 cggtgccgga accctcccaa

2761 ttcccctccc tggccccaca tccccaaccc tgctgctgga
 gtgctctcgc aaaccctgc
 2821 tgctgcctgg aaaaaagtgc ccaagctgct gacgcaaaaa g

**5 Sequence ID No: 4 - mouse espin isoform 1 amino acid sequence
 (NCBI Reference Sequence: NP_997570.1)**

1 maleqalqaa rrgdldvlrs lhaagllgps lrdslldalpv hhaarsgklh
 clrylveeva
 10 61 lpavsrarn g atpahdaaat gylsclqwl tgggcrvqek dnsgatvlhl
 aarfghpdvv
 121 kwlllyqggan saitttdtgal pihyaaakgd lpslklvlgh ypegvnaqtn
 ngatplylac
 181 qeghlevtky lvqecsadph lraqdgmtp l haaaqmghnp vlvwlvsfad
 15 vsfseqdhdg
 241 atamhfaasr ghtkvlswll lhgaeisqdl wggtplhdaa engeleccqi
 lavngagldv
 301 rdhdgytaad laefnghthc srylrvqtl slehrvlrd qsmdleakql
 dsgmsspntt
 20 361 msvqpmtdl gsptstfsny dscssshsss kgqrsnrgip garaadlqsy
 mdmlnpeksl
 421 prgklgkps ppppppppps fppppptgt qpppppgyp apnppvghl
 nniymqtknk
 481 lrhvevds lk epkvelndqf aqpssgdghs glhrqdsgll rqdsellhrq
 25 ellrhstglr
 541 rqdskrkrs fskqpstgdy yrqlgrspge plaarpgmah seeaallpgn
 hvhngcsads
 601 kasrelpppp pppplpeals spppapplpi egagaacgqr rsssstgkvr
 vlrhrkstks
 30 661 fnmmsptgdn sellaeikag kslkptpqsk glttvfsgsg qpasqpespq
 plvspapsrt
 721 rsptppasgs qpllngsvvp appatpapg v hldvealip ldeqgrpipe
 wkrqvmvrkl
 781 qqkmqeeeq rrkeeeear laslpawrrd ilrkkleer eqkrkeeerq
 35 kleeigrake
 841 qseklrtlg y deaklapwqr qvilkkgeip k

**Sequence ID No: 5 - Mus musculus espin (Espn), transcript
 variant 4, mRNA (NCBI Reference Sequence: NM_207689.2)**

40 1 agagaggagg aggaggagca ggcttgccgg tagcagggac
 agcatgggca atagcttga
 61 acaccgagtc ttgtcccggg atcaatccat ggacctggag
 gcaaagcagc tggactcggg
 45 121 tatgtcctcg cccaacacca ccatgtcggc ccagccaatg
 acctttgacc tgggctcgcc
 181 tactagcacg ttctccaact atgactcctg ctctccagt
 cactccagca gcaaggggca
 241 gcgatcgaat cgagggattc caggtgcaag agctgcagac
 50 ttacagagct acatggacat

301 gctgaaccca gagaagagct tgcctcgggg caagctaggg
 aagccttccc cgccaccacc
 361 tccaccacca ccaccaccaa gcttcccgcc acccccacca
 cccacaggca cccagccgcc
 5 421 cccacctcca ccaggctacc cagctcccaa tccccctgtg
 ggactgcatc tgaataacat
 481 ttacatgcag accaagaaca agcttcgcca tgtggagggtg
 gactcgctca aggaggcggc
 541 gctgctcccc gggaaccacg tgcacaacgg ctgctcagcg
 10 gactccaaag cgtccaggga
 601 gctgccgccg ccaccgccgc cgccgccgct gcccgaggcc
 ctgagttcgc cgccgccgc
 661 cccacctctg cccatcgagg gcgcggggcg agcctgcggg
 cagcgtcgtt cctcgtcttc
 15 721 tactggcaaa gtgagagtcc tgagacacag gaagagcacc
 aaatctttca acatgatgtc
 781 cccaacgggt gataactcag agcttctggc tgagataaag
 gcgggcaaga gcctgaagcc
 841 gacaccgcag agcaaggggc tgacaaccgt gttctcaggc
 20 agtgggcagc cagcctccca
 901 gcctgagtca ccgcagcctc tgggtgtcacc tgcgccatct
 cggaactcga gccccacccc
 961 gccagcctct gggctctcagc cactgctcaa tggcagtgtg
 gtgccggcac cacctgccac
 25 1021 cccggcacct ggagtccatc tggatgtgga ggccctcatt
 cccactcttg atgagcaggg
 1081 ccggcccatc ccggagtgga agcgccagggt gatggctccgc
 aagctgcagc agaagatgca
 1141 ggaggaagag gagcagcgga ggaaggagga agaggaggag
 30 gcccggtctg ccagcctgcc
 1201 tgcctggaga cgagacattc ttcggaagaa gctggaggag
 gagagggagc agaagcgaaa
 1261 agaggaggag cggcaaaagc tggaggaaat acagagggcg
 aaagaacagt cggagaagct
 35 1321 gcggacacta ggctacgacg aagccaagct cgcgccctgg
 cagcgacagg tcatcttgaa
 1381 gaagggggag atccctaagt aataggagtc tctcggcttc
 ttgcgtgtag cctcacaagt
 1441 tctgagagat ggggagcggc cccagcccc cgccccatcc
 40 tgtcaggttg gtgcggaagg
 1501 gctaggatcc ttgccgcct tcccttcggt gccggaaccc
 tccaattcc cctccctggc
 1561 cccacatccc caaccctgct gctggagtgc tctcgcaaac
 ccctgctgtc gcctggaaaa
 45 1621 aagtgcccaa gctgctgacg caaaaag

Sequence ID No: 6 - mouse espin isoform 4 amino acid sequence
(NCBI Reference Sequence: NP_997572.1)

50 1 mgnslehrvl srdqsmlea kqldsgmssp ntmsvqpmt
 fdlgststf snydscsssh

61 ssskgqrsnr gipgaraadl qsymdmlnpe kslprgklgk
 psppppppppp ppsfpppppp
 121 tgtqpppppp gypapnppvg lhlnniymqt knklrhvevd
 slkeaallpg nhvhngcsad
 5 181 skasrelppp ppppplpeal ssppppapplp iegagaacgq
 rrsssstgkv rvlrhrkstk
 241 sfnmmsptgd nsellaeika gkslkptpqs kglttvfsgs
 ggpasqpesp qplvspapsr
 301 trsptppasg sqpllngsvv pappatpapg vhldvealip
 10 tldeqgrpip ewkrqvmvrk
 361 lqqkmqeeee qrrkeeeeee rlaslpawrr dilrkkleee
 reqkrkeeer qkleeiqrak
 421 eqseklrtlg ydeaklapwq rqvilkkgei pk
 15 **Sequence ID No: 7 - rat espin (Espn), transcript variant 1,
 mRNA (NCBI Reference Sequence: NM_019622.2)**

1 gccgaggggtg acaccatggc cctggaacag gcgatgcagg
 cggcacggcg gggcgacctg
 20 61 gacgtgctga ggtccctgca cgccgccggc ctgctggggc
 cttctctgcg cgacccgcta
 121 gacgccctgc cgtgacacca tgcggcccgc tcaggcaagc
 tgcactgttt gcgctacttg
 181 gtggaggagg ttgccctccc agctgtgtcc cgcgcgcgca
 25 acggcgccac accagcccat
 241 gatgccgctg ccacgggcta cctctcttgc ctgcagtggc
 tgctcacaca ggggtggctgc
 301 aggggtgcagg aaaaagataa ctctgggtgcc acggtcctgc
 acctggctgc ccgctttggc
 361 caccgcgacg tggatgaactg gctgctgtac cagggcggtg
 cgaactctgc catcaccaca
 421 gacacgggcg ccctgcctat ccactatgct gccgccaaag
 gagatctccc ctccatgaag
 481 cttcttgtcg ggcactaccc tgaggagtg aatgcccaaa
 35 ccaacaacgg tgccacgccc
 541 ctgtacctgg cgtgccagga gggccacctg gaagtgcga
 agtaccttgt gcaggagtgc
 601 agtgcagatc cgcacctgcg cgcccaagac ggcacgacct
 ccctgcatgc cgcggcgcag
 661 atggggccaca acccagtcct ggtgtggctg gtgagctttg
 cggacgtgag cttttcggag
 721 caggaccacg acggcgccac agccatgcac tttgcagcca
 gccgcggcca caccaaagt
 781 ctacgtggc tcctgctgca cggcgagag atctcccagg
 45 acctgtgggg cgggaccccc
 841 ctgcatgatg ctgctgagaa cggggaactg gactgctgcc
 agatcctcgc ggtgaatggt
 901 gcgggggctg acgtccgcga ccacgatggg tatacggctg
 cggacctggc tgatttcaat
 961 ggccacaccc actgctcccg ctacctacgt acggtgcaaa
 50 ccctgagctt ggaacaccga

1021 gtcctgtccc gggatccatc catggacctg gaggcaaagc
 agccggactc aggcattgtc
 1081 tcaccaaca ccaccatgtc ggtccagccg ccgaactttg
 accttggtc acccaccagc
 5 1141 accctctcca actatgactc ctgctcctcc agccattcca
 gcagcaaggg tcagcgctct
 1201 actcgagggt ccagatcctc agacttacag agctacatgg
 acatgctgaa cccagagcct
 1261 cggagcaagc aagggaagcc ttcattctta ccaccaccgc
 10 caccaccaag ctccctcca
 1321 ccaccacccc caggcacaca gctgccccca cctccaccag
 gctaccagc tccaatccc
 1381 cctgtggggc tgcatttgga taacatttac atgcagacca
 agaacaaact ccgccacgtg
 15 1441 gaggtggact ccctcaagaa ggagccgagc tccggcgagc
 gctactcggg gctacgcagg
 1501 caggattccg ggctgctcag gcaggattcg gagctgctgc
 tcaggcaca caccggactg
 1561 cgcaggcagg actccgaccg caaacagcgt tcattcagta
 20 aacagcccag cacgggggac
 1621 tactaccgcc agctggggccg cagcccgggg gagccgctgg
 ccgcacgcc gggcatggcc
 1681 cacagcgagg aggcggcgct gctccccggg aaccacgtgc
 acaacggctg ctccgcggac
 25 1741 tccaaagcgt ccaggagct gccgccgcca ccgccgccgc
 cgcccctgcc cgaggccctg
 1801 agttcgccgc caccgcccc acctctgccc atcgagggcg
 cgggcgcagc ctgcggacag
 1861 cggcgcttct catctttctac tggcagcacc aaatctttca
 30 acatgatgtc cccaacgggt
 1921 gacaactcgg agttgctggc tgaaataaaa gcaggcaaga
 gtctgaaacc aacaccgcag
 1981 agcaaggggc tgacaacagt gttctcaggc agcgggcagc
 cagcctccca gcctgagtca
 35 2041 ccacagcctg cgggtgtcacc tgggccatct cgggcccggg
 gccccacccc accagcctct
 2101 gggcctcagc cactgctcaa tggcagcata gtaccggcac
 cacctgccac cctagcacca
 2161 ggagtgcac tggtgtgga ggccctcatc cccacacttg
 40 atgagcaggg ccggcccatc
 2221 ccggagtgga agcggccagg gatgggtccg aagctgcagc
 agaaaatgca ggaggaagag
 2281 gagcagcgga ggaaggaaga agaggaggag gcccggtgg
 ccagcctgcc cgctggaga
 45 2341 cgagacattc ttaggaagaa gctggaggag gagagggagc
 agaagcgaaa agaggaggag
 2401 cggcagaagc tggaggaaat ccagagggcg aaagaacagt
 cggagaagct gcggacacta
 2461 ggatacgac aggccaaagt cgcgccctgg cagcgacagg
 50 tcattcttgaa gaagggggag

2521 atccctaagt aataggagtc tcggcctctt gcgtgtagcc
 tcacgagctc tgagaaatgg
 2581 ggagcggccc ccagcccccg ccccatcctg tcaggttggt
 gcggaagggt gggagccttg
 5 2641 agcccttccc ttcggtgcct gccggaatct tccctattcc
 cctccctggc cccacatccc
 2701 caaccctgct gctggagtg ctcgcgaac ccctgctgtc
 gcctggaaaa aaaaagtgcc
 2761 caggctgctg acgcaaaaag aaaaaaagag aaagaaaaaa
 10 gatgctcatt tgcatgctca
 2821 cttacatata tttgcatggt caccgaccca gccggagctc
 gccccagcct cgtgggtggt
 2881 gacttttctt gcagggcgca cgccctgctg cagccccctc
 ccccgagcc cggaaccac
 15 2941 attgctggcg catcctgggt ggaggcagac cccgagctcg
 gggaagggt tttcctccct
 3001 ccacgaccca gatctgtgct ggccaaagt gctgcgtttc
 ttatcccctg cgagggtttc
 3061 ctctcagtca tttgtttacc agaaaggatg aaacttgcct
 20 gtaggggagg agctgtcgt
 3121 cctgaggggc cccaccttta cacgtaccac cccctcccc
 cgccccagtt acgccttcca
 3181 agttcccagt caggccctta gcttccctga ccctttcact
 gcctcaatgg tataagtga
 25 3241 gggccaggga tcccgcaatt gtacctgctc ctcaccctat
 cccccacccc cttccttccc
 3301 ccattaaacc tctatccgcc aa

Sequence ID No: 8 - rat espin isoform 1 amino acid sequence
(NCBI Reference Sequence: NP_062568.2)

1 maleqamqaa rrgdldvlrs lhaagllgps lrdpldalpv
 hhaarsgklh clrylveeva
 61 lpavsrarng atpahdaaat gylsclqwill tgggcrvqek
 35 dnsgatvlhl aarfghpdvv
 121 nwlllyqggan saitttdtgal pihyaaakgd lpsmkllvgh
 ypegvnaqtn ngatplylac
 181 qeghlevtky lvqecsadph lraqdgmtp1 haaaqmghnp
 vlvwlvsfad vsfseqdhdg
 40 241 atamhfaasr ghtkvlswll lhgaeisqdl wggtp1hdaa
 engeleccqi lavngagldv
 301 rdhdgytaad ladfngthc srylrvqtl slehrvlrd
 psmdleakqp dsgmsspntt
 361 msvqppnfdl gsptstlsny dscssshsss kgqrstrgar
 45 ssdlqsymdm lnpeprskqg
 421 kpsslppppp psfppppppg tqlpppppgy papnppvgh
 ldniymqtkn klrhvevds1
 481 kkepssgdgy sglrrqdsgl lrqdselllr hntglrrqds
 drkqrsfskq pstgdyyrql
 50 541 grspgeplaa rpgmahseea allpgnhvhn gcsadskasr
 elpppppppp lpealssppp

601 applpiegag aacgqrrsss stgstksfnm msptgdnsel
 laeikagksl kptpqskglt
 661 tvfsgsgqpa sqpesqpav spgpsrarsp tppasgpqpl
 lngsivpapp atlapgvhld
 5 721 vealptlde qgrpipewkr qvmvrklqqk mqeeeeeqrk
 eeeeearlas lpawrrdilr
 781 kkleereqk rkeeerqkle eiqrakeqse klrtlgydea
 klapwqrqvi lkkgeipk

10 **Sequence ID No: 9 – rat espin (Espn), transcript variant 4,
 mRNA (GenBank: AF540947.1; GI:28822427)**

1 atgggtgata gcttgggaaca ccgagtcctg tcccgggatc
 catccatgga cctggaggca
 15 61 aagcagccgg actcaggcat gtcttcaccc aacaccacca
 tgtcgggtcca gccgccgaac
 121 ttgaccttg gctcaccac cagcaccctc tccaactatg
 actcctgctc ctccagccat
 181 tccagcagca agggtcagcg ctctactcga ggtgccagat
 20 cctcagactt acagagctac
 241 atggacatgc tgaaccacaga gcctcggagc aagcaaggga
 agccttcac tctaccacca
 301 ccgccaccac caagcttccc tccaccacca cccccaggca
 cacagctgcc cccacctcca
 25 361 ccaggctacc cagctcccaa tccccctgtg gggctgcatt
 tggataacat ttacatgcag
 421 accaagaaca aactccgcca cgtggagggtg gactccctca
 agaaggagcc gagctccggc
 481 gacggctact cggggctacg caggcaggat tccgggctgc
 30 tcaggcagga ttcggagctg
 541 ctgctcaggc acaacaccgg actgcgagc caggactccg
 accgcaaaca gcgttcattc
 601 agtaaacagc ccagcacggg ggactactac cgccagctgg
 gccgcagccc gggggagccg
 35 661 ctggccgcac gcccgggcat ggcccacagc gaggaggcgg
 cgctgctccc cggaaccac
 721 gtgcacaacg gctgctccgc ggactccaaa gcgtccaggg
 agctgccgcc gccaccgccg
 781 ccgccgcccc tgcccagggc cctgagttcg ccgccaccgg
 40 cccacctct gcccatcgag
 841 ggcgcgggcg cagcctgcgg acagcggcgt tcctcatctt
 ctactggcaa agtgagaatc
 901 ctgagacaca ggaagagcac caaatctttc aacatgatgt
 ccccaacggg tgacaactcg
 45 961 gagttgctgg ctgaaataaa agcaggcaag agtctgaaac
 caacaccgca gagcaagggg
 1021 ctgacaacag tgttctcagg cagcgggcag ccagcctccc
 agcctgagtc accacagcct
 1081 gcggtgtcac ctggggccatc tcggggcccg agccccaccc
 50 caccagcctc tgggcctcag

```

1141 ccactgctca atggcagcat agtaccggca ccacctgcca
ccctagcacc aggagtgcac
1201 ctggatgtgg aggccctcat cccacactt gatgagcagg
gccggcccat cccggagtgg
5 1261 aagcgccagg tgatgggtccg gaagctgcag cagaaaatgc
aggaggaaga ggagcagcgg
1321 aggaaggaag aagaggagga ggcccggctg gccagcctgc
ccgcctggag acgagacatt
1381 cttaggaaga agctggagga ggagagggag cagaagcgaa
10 aagaggagga gcggcagaag
1441 ctggaggaaa tccagagggc gaaagaacag tcggagaagc
tgcggacact aggatacgac
1501 gaggccaaagc tcgcgccctg gcagcgacag gtcattctga
agaaggggga gatccctaag
15 1561 taa

```

**Sequence ID No: 10 – rat espin isoform 4 amino acid sequence
(GenBank: AA050331.1; GI:28822428)**

```

20 1 mgdslehrvl srdpsmdlea kqpdsgmssp nttmsvqppn
fdlgsptstl snydscsssh
61 ssskgqrstr garssdlqsy mdmlnpeprs kqgkpsslpp
ppppsfpfppp ppgtqlpppp
121 pgypapnppv glhldniymq tknklrhvev dslkkepssg
25 dgysglrrqd sgllrqdsel
181 llrhntglrr qdsdrkqrsf skqpstgdyr rqlgrspgpe
laarpgmahs eeaallpgnh
241 vhnngcsadsk asrelppppp ppplpealss pppapplpie
gagaacgqrr sssstgkvri
30 301 lrhrkstksf nmmsptgdns ellaeikagk slkptpqskg
lttvfsgsgq pasqpespqp
361 avspggsrar sptppasgpq pllngsivpa ppatlapgvh
ldvealipth deqgrpipew
421 krqvmvrklq qkmqeeeeqr rkeeeeeearl aslpawrrdi
35 lrkkleeere qkrkeeerqk
481 leeiqrakeq seklrtlgdy eaklapwqrq vilkkgeipk

```

CLAIMS

What is claimed is:

1. A method of inducing stereocilia and/or stereociliary bundles in an
5 inner ear auditory hair cell in a subject in need thereof comprising administering to and
expressing within the hair cell in the inner ear a polynucleotide encoding espin isoform 1
(ESPN1).
2. The method of claim 1, wherein the subject is human.
3. A method of inducing stereocilia and/or stereociliary bundles in an
10 inner ear auditory hair cell comprising administering to and expressing within the hair cell a
polynucleotide encoding espin isoform 1 (ESPN1).
4. The method of claim 3, wherein the hair cell is *in vivo*.
5. The method of claim 3, wherein the hair cell is *in vitro*.
6. The method of any one of claims 1 to 5, wherein the hair cell is a
15 damaged or regenerated hair cell.
7. The method of claim 6, wherein the hair cell has been damaged by
exposure to an aminoglycoside.
8. The method of any one of claims 1 to 7, wherein the polynucleotide
encoding ESPN1 is expressed under the control of a promoter heterologous to the
20 polynucleotide.
9. The method of any one of claims 1 to 8, wherein the polynucleotide
encoding ESPN1 is administered to the hair cell in a viral vector.
10. The method of claim 9, wherein the viral vector is from a virus
selected from the group consisting of adenovirus, adeno-associated virus, lentivirus, and
25 retrovirus.
11. The method of claim 10, wherein the viral vector is replication
defective.

12. The method of any one of claims 1 to 8, wherein the polynucleotide encoding ESPN1 is administered to the hair cell in a plasmid vector.

13. The method of any one of claims 1 to 12, wherein the polynucleotide encoding ESPN1 has at least 60% sequence identity to one or more of SEQ ID NOs: 1, 3, 5,
5 7 or 9.

14. The method of any one of claims 1 to 13, further comprising administering to the hair cell a polynucleotide encoding ATOH1 (atonal bHLH transcription factor 1).

15 10 15. The method of any one of claims 1 to 14, further comprising administering to the hair cell a γ -secretase inhibitor.

16. The method of claim 15, wherein the γ -secretase inhibitor is DAPT (N-[N-[(3,5-Difluorophenyl)acetyl]-L-alanyl]-L-phenylglycine-tert-butyl).

17. The method of any one of claims 1 to 16, wherein the induced stereociliary bundles comprise a stair-step arrangement and a single, central kinocilium.

15 18. The method of any one of claims 1 to 17, wherein the induced stereocilia in a stereociliary bundle are linked at their tops.

19. The method of any one of claims 1 to 18, wherein the induced stereociliary bundles exhibit functional mechanoelectrical transduction channels.

20 20. A method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell in a subject in need thereof comprising administering to the hair cell in the inner ear an ESPN1 polypeptide.

21. The method of claim 20, wherein the subject is human.

22. A method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell comprising administering to the hair cell an ESPN1 polypeptide.

25 23. The method of claim 22, wherein the hair cell is *in vivo*.

24. The method of claim 22, wherein the hair cell is *in vitro*.

25. The method of any one of claims 20 to 24, wherein the hair cell is a damaged or regenerated hair cell.

26. The method of claim 25, wherein the hair cell has been damaged by exposure to an aminoglycoside.

5 27. The method of any one of claims 20 to 26, wherein the ESPN1 polypeptide is administered in a nanoparticle, a liposome and/or a microparticle.

28. The method of any one of claims 20 to 27, wherein the ESPN1 polypeptide has at least 60% sequence identity to one or more of SEQ ID NOs: 2, 4, 6, 8 or 10.

10 29. The method of any one of claims 20 to 28, further comprising administering to the hair cell a polynucleotide encoding ATOH1 (atonal bHLH transcription factor 1).

30. The method of any one of claims 20 to 29, further comprising administering to the hair cell a γ -secretase inhibitor.

15 31. The method of claim 30, wherein the γ -secretase inhibitor is DAPT (N-[N-[(3,5-Difluorophenyl)acetyl]-L-alanyl]-L-phenylglycine-tert-butyl).

32. The method of any one of claims 20 to 31, wherein the induced stereociliary bundles comprise a stair-step arrangement and a single, central kinocilium.

20 33. The method of any one of claims 20 to 32, wherein the induced stereocilia in a stereociliary bundle are linked at their tops.

34. The method of any one of claims 20 to 33, wherein the induced stereociliary bundles exhibit functional mechanoelectrical transduction channels.

35. An otic solution comprising a polynucleotide encoding espin isoform 1 (ESPN1).

25 36. The otic solution of claim 35, wherein the polynucleotide is in a viral vector or a plasmid vector.

37. The otic solution of any one of claims 35 to 36, wherein the viral vector is from a virus selected from the group consisting of adenovirus, adeno-associated virus, lentivirus, and retrovirus.

5 38. The otic solution of claim 37, wherein the viral vector is replication defective.

39. The otic solution of any one of claims 35 to 38, wherein the polynucleotide encoding ESPN1 has at least 60% sequence identity to one or more of SEQ ID NOs: 1, 3, 5, 7 or 9.

40. An otic solution comprising an ESPN1 polypeptide.

10 41. The otic solution of claim 40, wherein the ESPN1 polypeptide has at least 60% sequence identity to one or more of SEQ ID NOs: 2, 4, 6, 8 or 10.

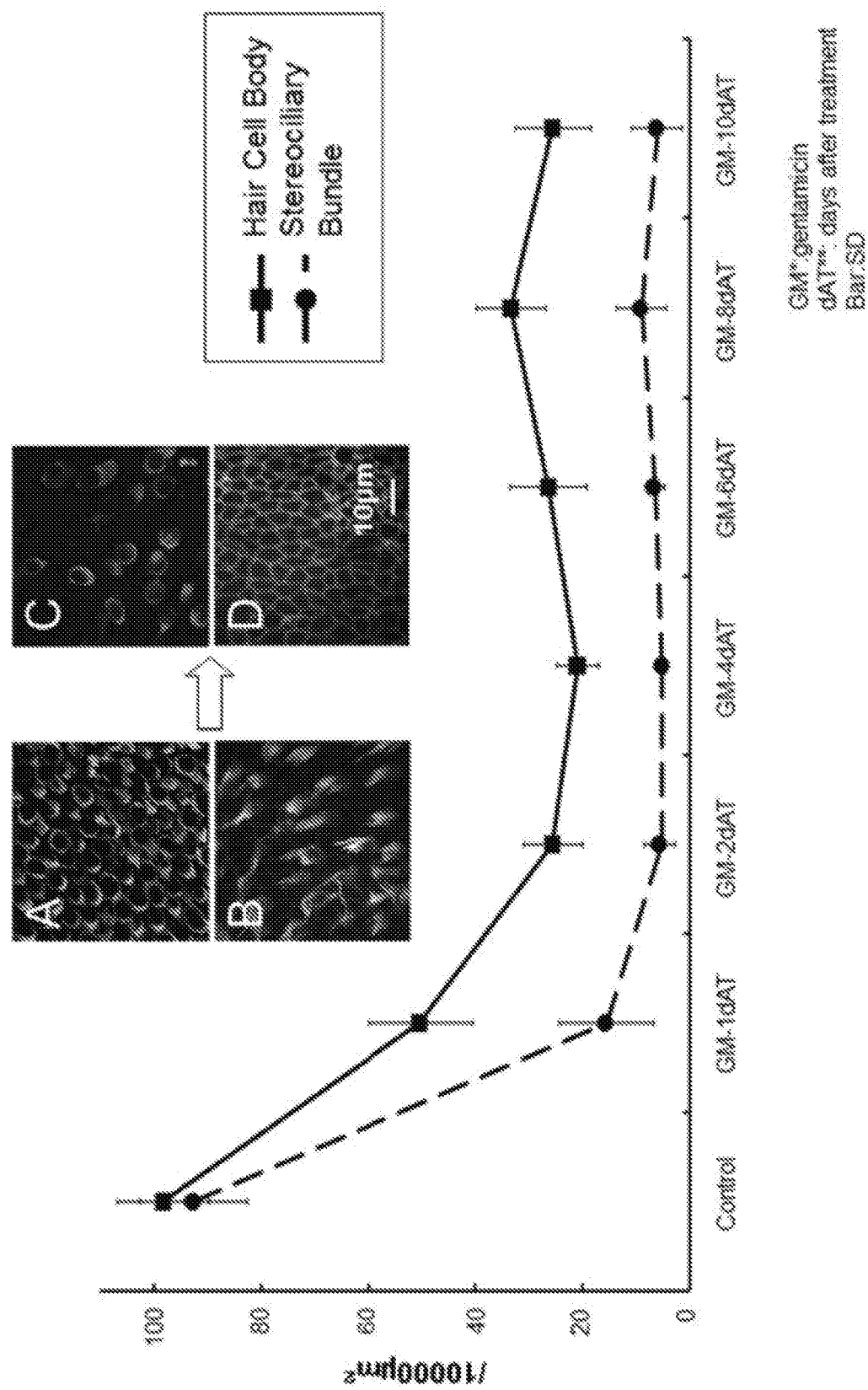
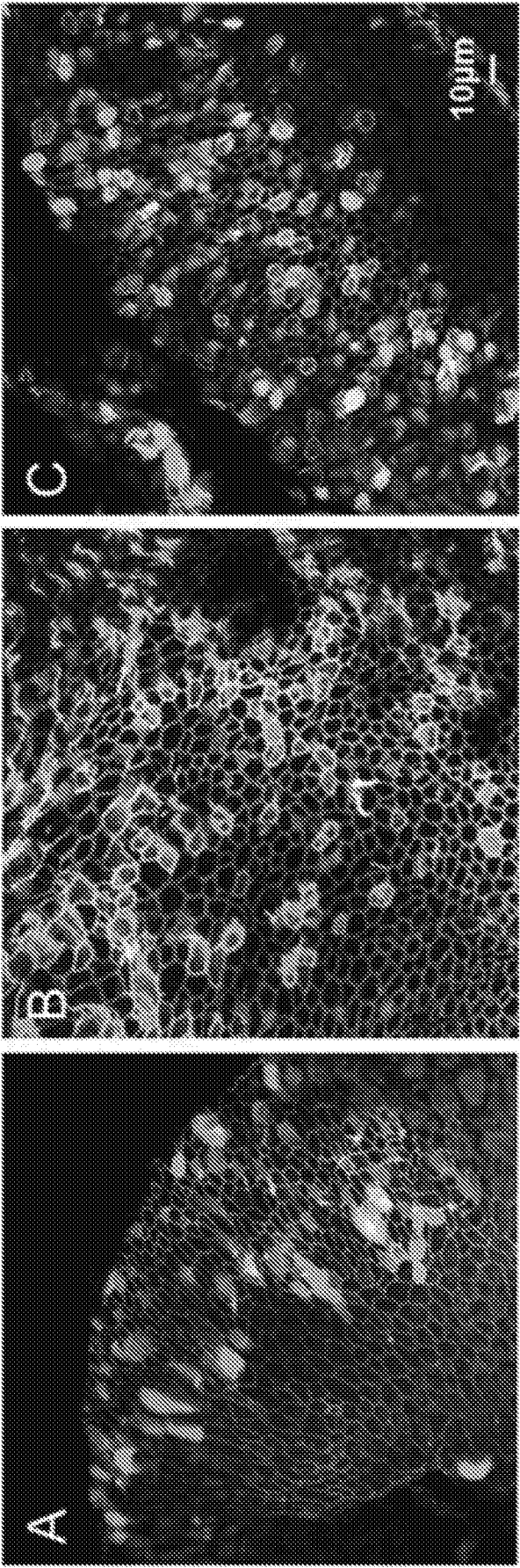


Fig. 1A-D



Red: Myosin7A, Green: GFP, White: phalloidin, Blue: DAPI

Fig. 2A-C

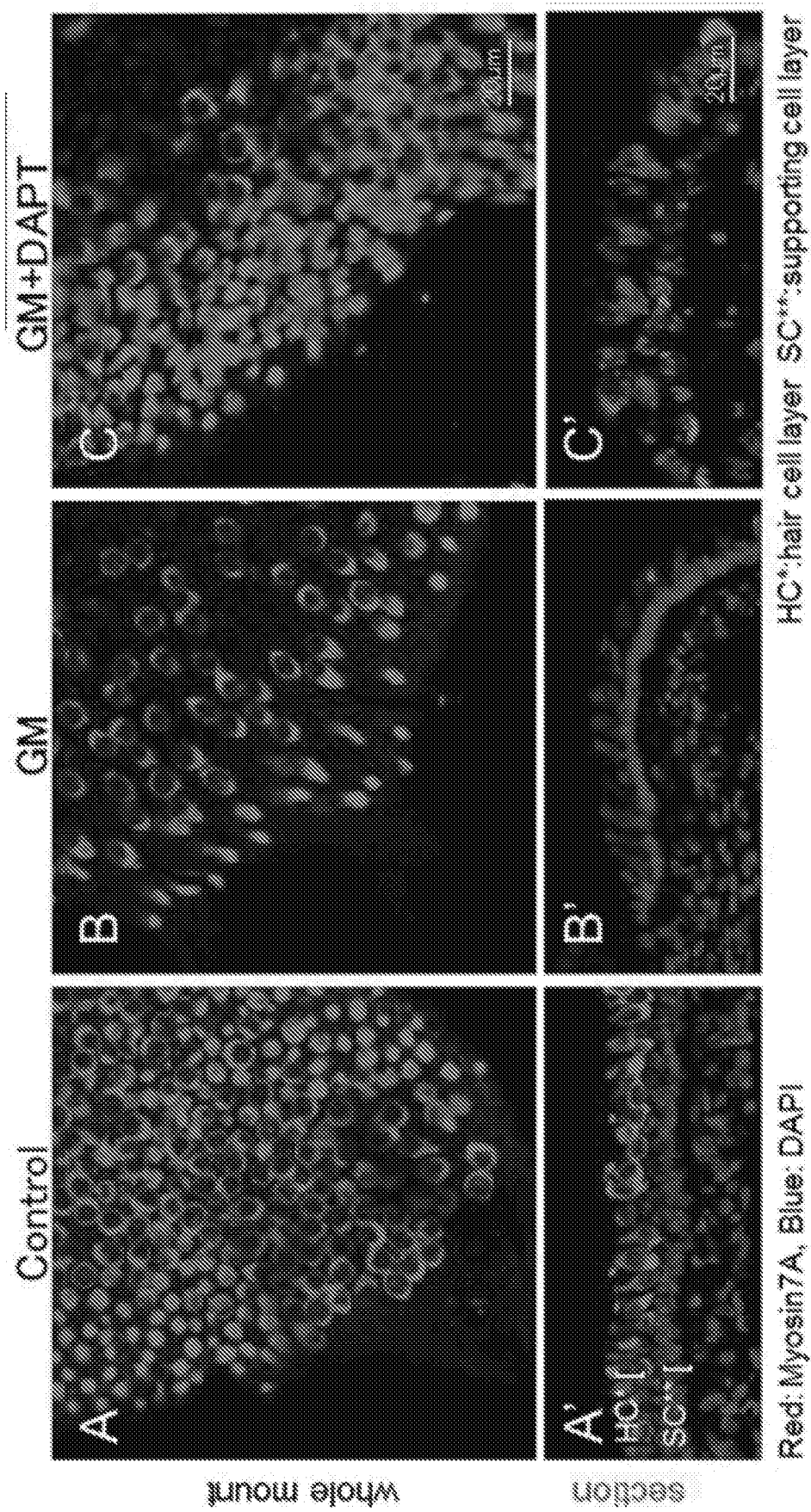


Fig. 3A-C

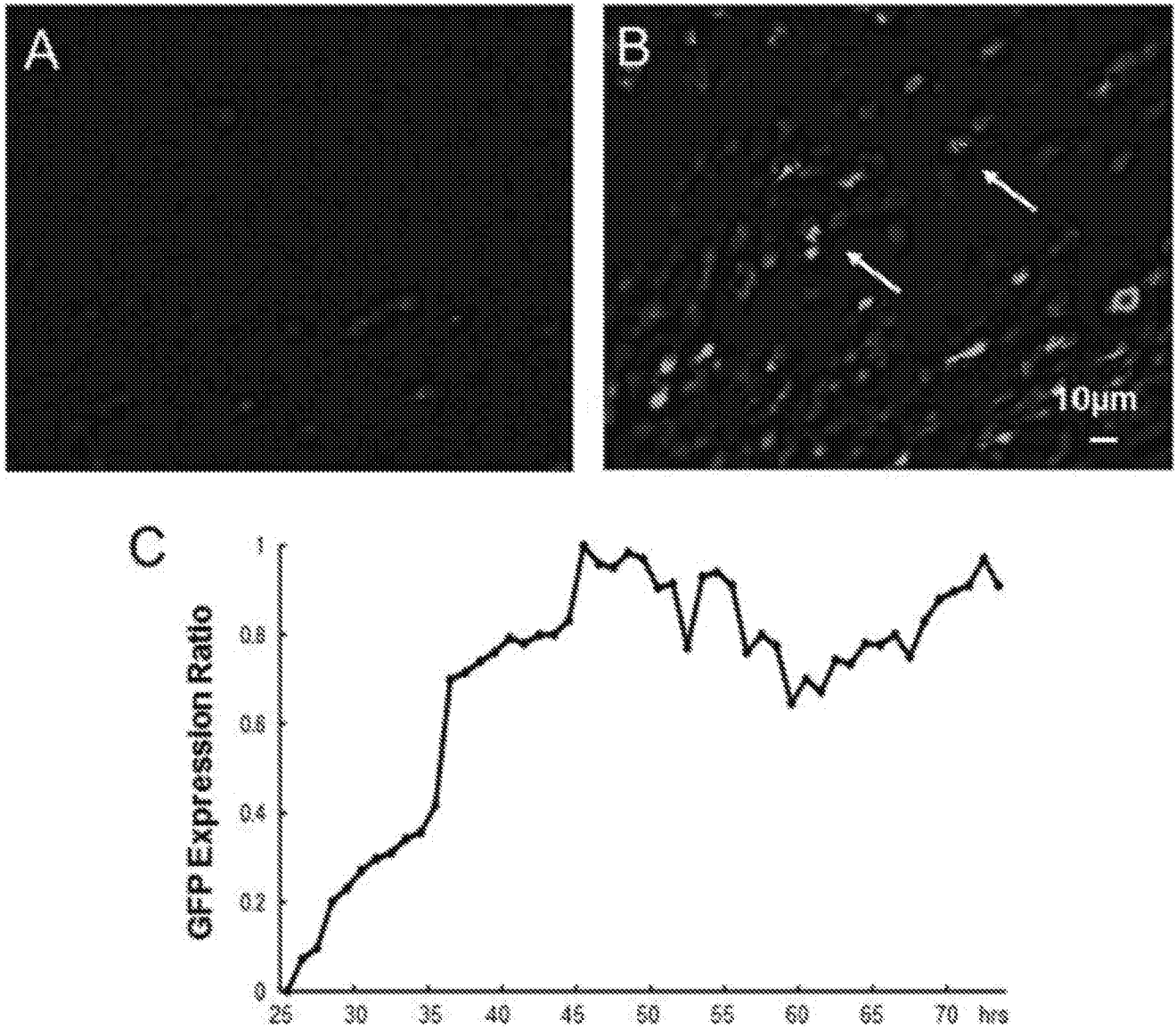


Fig. 4A-C

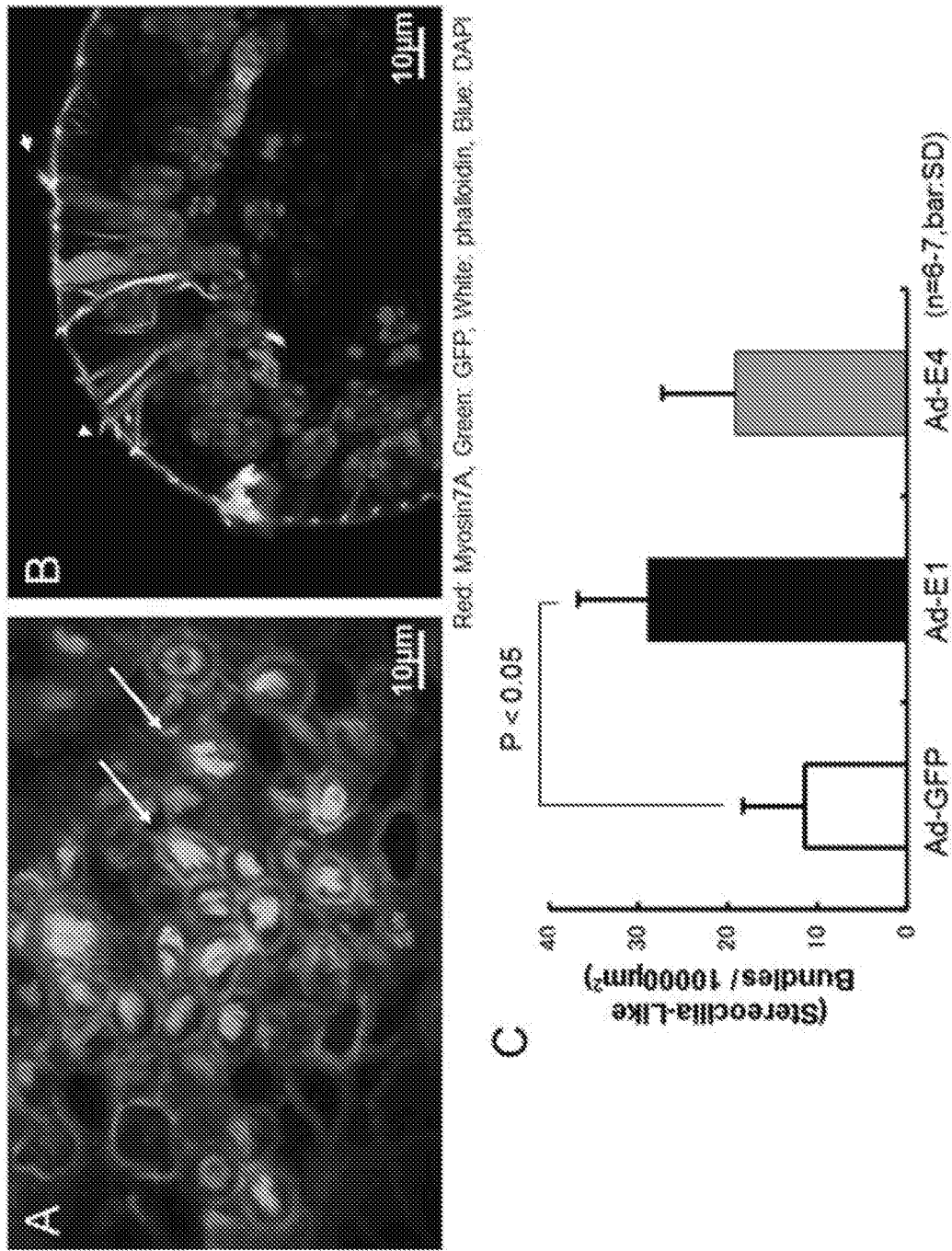


Fig. 5A-C

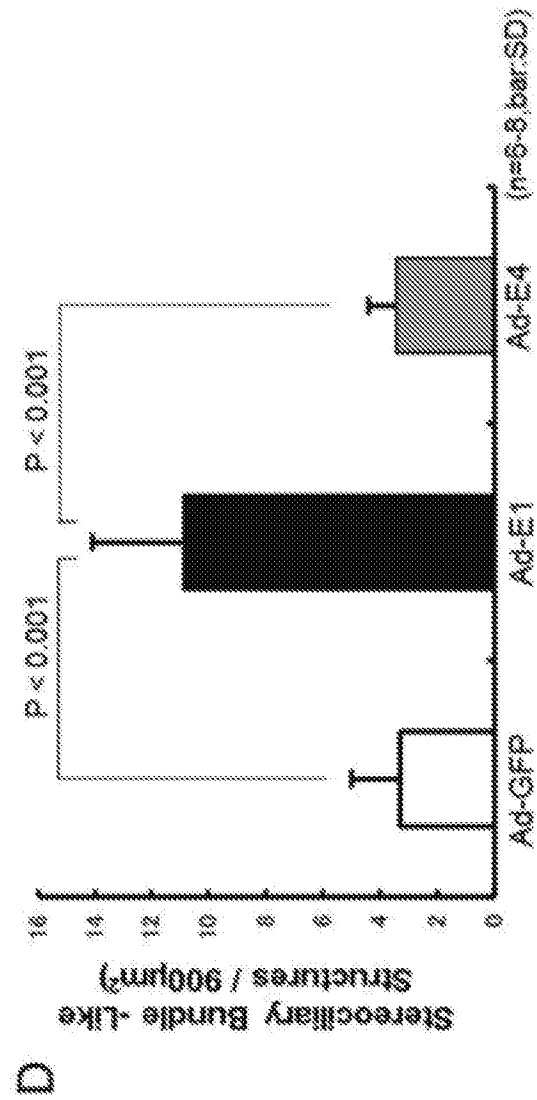
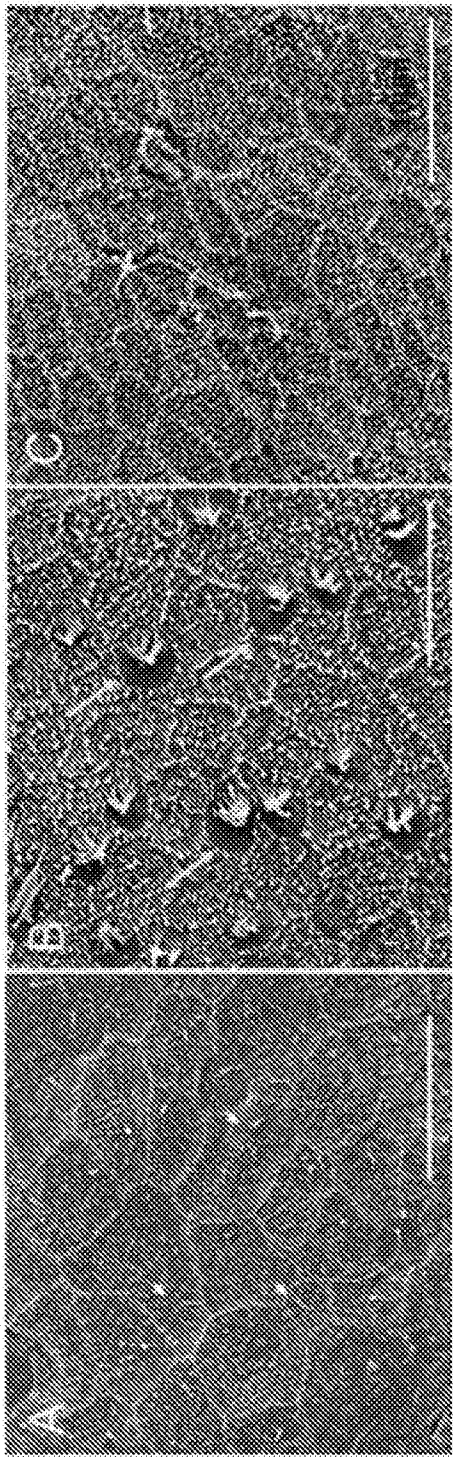


Fig. 6A-D

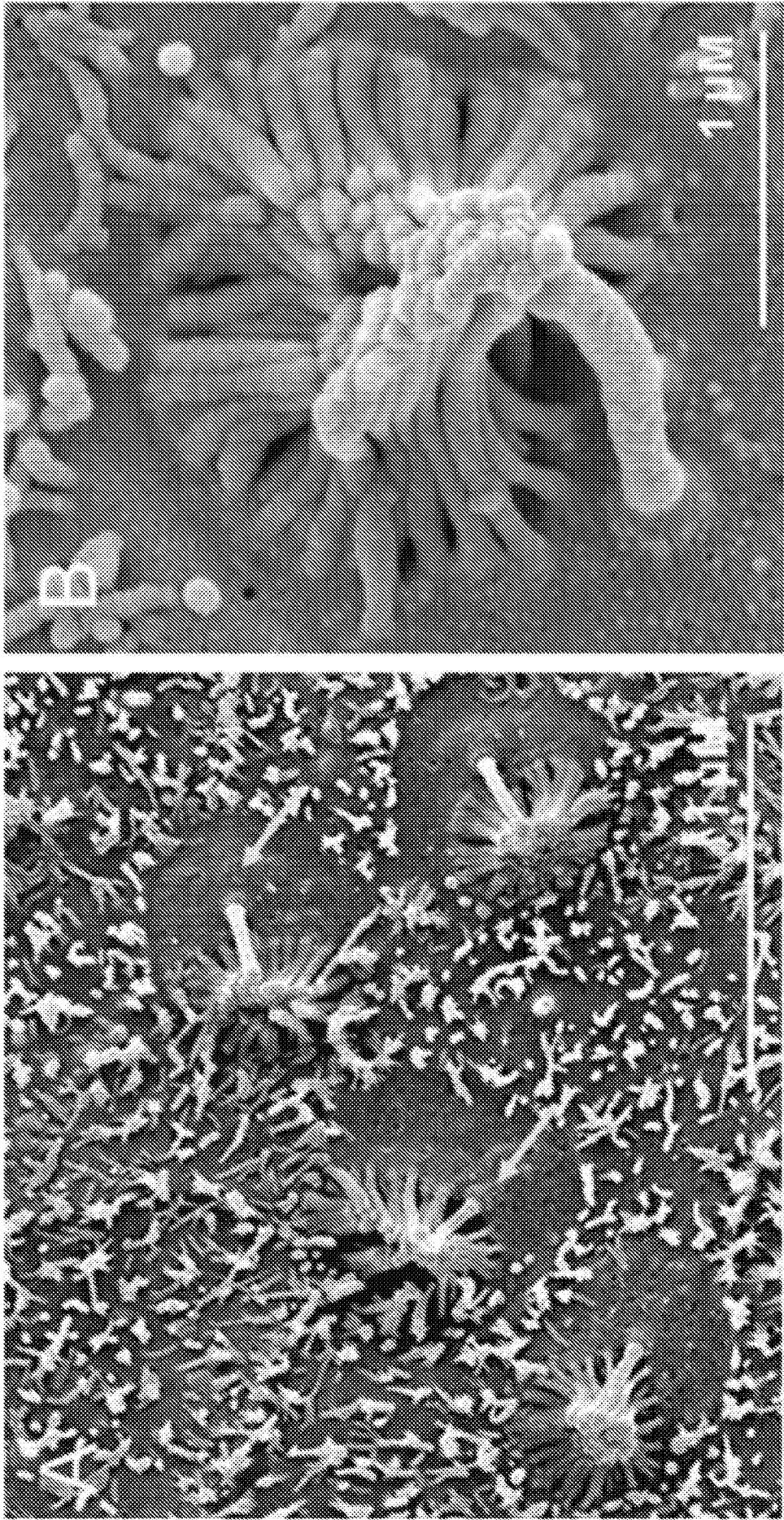


Fig. 7A-B

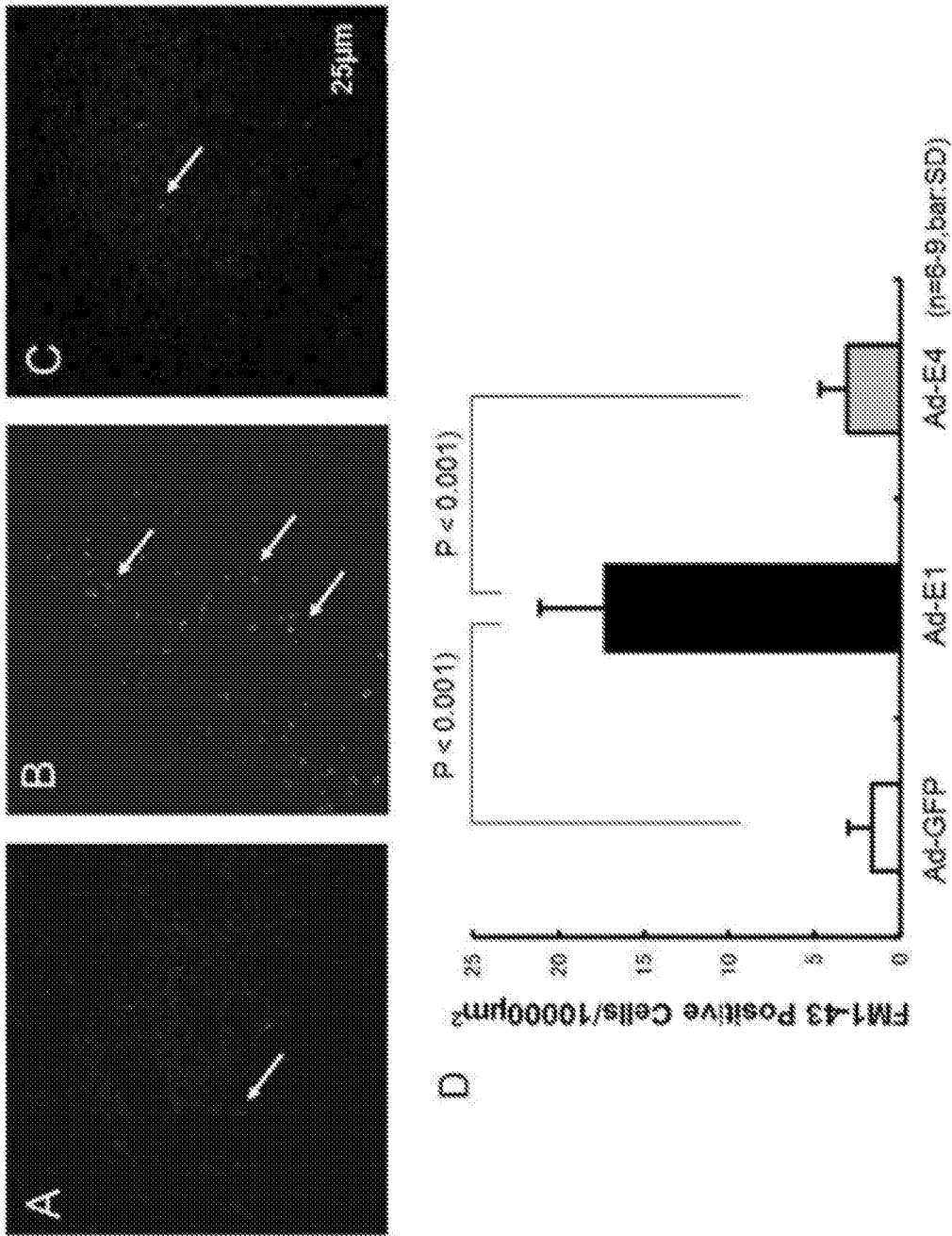


Fig. 8A-D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/43075

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/18, 9/00, 47/42 (2017.01)

CPC - A61K 9/0046, 38/18, 47/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---	TAURA, A, et al. Hair Cell Stereociliary Bundle Regeneration by ESPIN GENE Transduction After Aminoglycoside Damage and Hair Cell Induction by Notch Inhibition. Gene Ther. May 2016, Vol. 23, No. 5; Pages 415-423. DOI: 10.1038/gt.2016.12; Abstract; Page 2, Second Paragraph; Page 2, Third Paragraph; Page 3, Third Paragraph, Results section, Gentamicin damage to HC stereociliary bundles and cell bodies; Page 4, First Paragraph; Page 6, Fourth Paragraph; Page 7, First Paragraph; Page 7, Third Paragraph, Production of Ad-espin vectors section; Homo sapiens espin (ESPN), mRNA; NCBI Reference Sequence: NM_031475.2	1-3, 5, 6/1-6/3, 6/5, 7/6/1-7/6/3, 7/6/5, 20-22, 24, 25/20-25/22, 25/24, 26/25/20-26/25/22, 26/25/24, 35, 36, 37/35, 37/36, 38/37/36, 40 ----- 4, 6/4, 7/6/4, 23, 25/23, 26/25/23, 40, 41
Y	NARAYANA, P, et al. Length Regulation of Mechanosensitive Stereocilia Depends on Very Slow Actin Dynamics and Filament Severing Proteins. Nat Commun. 21 October 2015, Vol. 6; No. 6855. DOI: 10.1038/ncomms7855; Abstract	4, 6/4, 7/6/4, 23, 25/23, 26/25/23
Y	US 2016/0095902 A1 (OTONOMY, INC., et al.) 07 April 2016; Abstract; Claim 31	40, 41
Y	DONAUDY, F, et al. Espin Gene (ESPN) Mutations Associated with Autosomal Dominant Hearing Loss Cause Defects in Microvillar Elongation or Organisation. J Med Genet. 2006, Vol. 43; pages 157-161. DOI: 10.1136/jmg.2005.032086; Page 158, Left Column, First Paragraph; Page 158, Left Column, Results section; Table 1; Page 160, Right Column, Fifth Paragraph.	41

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 November 2017 (28.11.2017)

Date of mailing of the international search report

14 DEC 2017

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/43075

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 8-19, 27-34, 39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

---Continued within Next Supplemental Page---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

---Please See Supplemental Page---

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US17/43075

.-***-Continued from Box No. III: Observations Where Unity of Invention is Lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-7, 20-26, 35-38, 40-41; SEQ ID NO:2 (ESPN1 polypeptide) are directed toward methods and compositions for inducing functional stereocilia and stereocilia bundles in inner ear auditory hair cells comprising administering to and/or expressing in the hair cells sufficient levels of espin isoform 1 (ESPN1).

The methods and compositions will be searched to the extent that they encompass SEQ ID NO: 2 (first exemplary ESPN1 polypeptide). Applicant is invited to elect additional ESPN1 polypeptide(s), with specified SEQ ID NO: for each, to be searched. Additional ESPN1 polypeptide(s) will be searched upon the payment of additional fees. It is believed that claims 1-7, 20-26, 35-38, 40 and 41(in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 2 (ESPN1 polypeptide). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an ESPN1 polypeptide encompassing SEQ ID NO: 4 (first exemplary elected ESPN1 polypeptide).

No technical features are shared between the polypeptide sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell in a subject in need thereof comprising administering to and expressing within the hair cell in the inner ear a polynucleotide encoding espin isoform 1 (ESPN1); a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell comprising administering to and expressing within the hair cell a polynucleotide encoding espin isoform 1 (ESPN1); a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell in a subject in need thereof comprising administering to the hair cell in the inner ear an ESPN1 polypeptide; a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell comprising administering to the hair cell an ESPN1 polypeptide; an otic solution comprising a polynucleotide encoding espin isoform 1 (ESPN1); an otic solution comprising an ESPN1 polypeptide.

However, these shared technical features are previously shared by the publication entitled 'Hair Cell Stereociliary Bundle Regeneration by Espin Gene Transduction After Aminoglycoside Damage and Hair Cell Induction by Notch Inhibition' by Taura, et al. (hereinafter 'Taura') in view of US 2016/0095902 A1 to Otonomy, Inc. et al. (hereinafter 'Otonomy').

Taura disclose a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell (a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell; abstract) comprising administering to and expressing within the hair cell a polynucleotide encoding espin isoform 1 (ESPN1) (using viral vector for transduction of ESPN1 in hair cell (comprising administering to and expressing within the hair cell a polynucleotide encoding espin isoform 1 (ESPN1)); abstract; page 3, second paragraph, fourth paragraph; figure 2); a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell (a method of inducing stereocilia and/or stereociliary bundles in an inner ear auditory hair cell; abstract) comprising administering to the hair cell an ESPN1 polypeptide (using viral vector for transduction of ESPN1 in hair cell (comprising administering to and expressing within the hair cell in the inner ear a polynucleotide encoding espin isoform 1 (ESPN1)); abstract; page 3, second paragraph, fourth paragraph; page 7, third paragraph; figure 2); abstract; page 3, second paragraph, fourth paragraph; page 7, third paragraph; figure 2); a polynucleotide encoding espin isoform 1 (ESPN1) (abstract; page 7, third paragraph); an ESPN1 polypeptide (abstract; page 7, third paragraph).

Taura does not disclose an auditory hair cell in the inner ear of a subject; and an otic solution.

Otonomy discloses an auditory hair cell in the inner ear of a subject in need thereof (paragraphs [0087], [0393], [0420]; claim 1); and an otic solution (otic composition; claim 31).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the disclosure of Taura, to include an auditory hair cell in the inner ear of a subject and an otic solution, as disclosed to Otonomy, in order to provide superior gene therapy that can restore stereocilia on damaged or regenerated HCs.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Taura and Otonomy references, unity of invention is lacking.