Chapter 19

The Use of Human Wharton's Jelly Cells for Cochlear Tissue Engineering

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Abstract

Tissue engineering focuses on three primary components: stem cells, biomaterials, and growth factors. Together, the combination of these components is used to regrow and repair damaged tissues that normally do not regenerate easily on their own. Much attention has been focused on the use of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), due to their broad differentiation potential. However, ESCs and iPSCs require very detailed protocols to differentiate into target tissues, which are not always successful. Furthermore, procurement of ESCs is considered ethically controversial in some regions and procurement of iPSCs requires laborious transformation of adult tissues and characterization. However, mesenchymal stem cells are an adult stem cell population that are not ethically controversial and are readily available for procurement. Furthermore, mesenchymal stem cells exhibit the ability to differentiate into a variety of cell types arising from the mesoderm. In particular, human Wharton's jelly cells (hWJCs) are mesenchymal-type stem cells found in umbilical cords that possess remarkable differentiation potential. hWJCs are a highly desirable stem cell population due to their abundance in supply, high proliferation rates, and ability to differentiate into multiple cell types arising from all three germ layers. hWJCs are used to generate several neurological phenotypes arising from the ectoderm and are considered for engineering mechanosensory hair cells found in the auditory complex. Here, we report the methods for isolating hWJCs from human umbilical cords and non-virally transfected for use in cochlear tissue engineering studies.

Key words Mesenchymal stem cells, Human Wharton's jelly cells, ATOH1, Non-viral gene delivery, Nucleofection, Characterization

1 Introduction

In tissue engineering, both neuronal and sensory tissues, genetic manipulation, and tailored growth factor induction methods are popular for guiding pluripotent stem cells such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) toward a target phenotype [1–6]. In cochlear tissue engineering, methods developed by Hashino et al. [7–9] and Ito et al. [10, 11] have both seen success in using induction methods to form the pre-placodal epithelium and otic prosensory vesicles to induce the development

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of mechanosensory hair cells. However, while much focus has remained on ESCs and iPSCs for differentiating neuronal phenotypes, there is another stem cell type that has shown potential in engineering neuronal phenotypes.

Mesenchymal stem cells (MSCs) isolated from the bone marrow have long been a standard in tissue engineering for regenerating cartilage, bone, and adipose tissues [12–15]. In addition to bone marrow-derived MSCs, a mesenchymal-type stem cell isolated from the gelatinous connective tissue, Wharton's jelly, of the human umbilical cord, has shown great promise for tissue engineering applications. Human Wharton's jelly cells (hWJCs) are a highly desirable stem cell population due the fact that hWJCs are abundant in supply, ethically non-controversial, exhibit no risk to the donor, are highly proliferative, and have demonstrated differentiation potential similar to human bone marrow stem cells [16, 17]. In addition, hWJCs have shown a remarkable ability to differentiate toward additional phenotypes such as those arising from neuronal lineages with exposure to growth factors or manipulation of gene expression [18, 19]. hWJCs are amenable to non-viral gene delivery methods such as Nucleofection™ [20, 21], and have shown the capability to differentiate toward a mechanosensory phenotype, when genes are manipulated in the NOTCH pathway [22]. Thus, hWJCs are a reliable stem cell type worth investigating and exploring for applications focused on engineering sensory epithelium in the cochlear and vestibular systems.

The aim of this chapter is to provide insights into the protocols used for isolation, sub-culture, preservation, transfection, and associated analyses and characterization of hWJCs. The use of hWJCs in cochlear tissue engineering may prove advantageous for regenerating mechanosensory hair cells and studying mechanisms of development and damage to sensory epithelia.

2 Materials

Chemicals are at minimum Biological Grade quality, unless otherwise noted.

2.1 Isolation of hWJCs from the Umbilical Cord, Culture, and Sub-culture

1. 1× Phosphate buffered saline (PBS): Dissolve 1 PBS packet (Sigma-Aldrich, St. Louis, MO) in 1 L deionized water. Autoclave and sterifilter through a 1000-mL EMD Millipore Stericup™ Sterile Vacuum Filter Unit (Fisher Scientific, Pittsburgh, PA).
2. Wash Solution: Mix 2 % 100× Antibiotic-Antimycotic solution (Life Technologies, Grand Island, NY) in sterile 1× PBS from **step 1,** Subheading 2.1.

3. Digestion Medium: Dissolve 100 mg Type II Collagenase (Worthington-Biochem, Lakewood, NJ) in 49 mL of Dulbecco's Modified Eagle Medium (DMEM) with low glucose and pyruvate (Life Technologies). Sterifilter solution through 50-mL Steriflip-GV unit (EMD Millipore, Billerica, MA). Add enough (~0.5 mL) of penicillin-streptomycin (10,000 U/mL) (Life Technologies) to digestion medium to obtain a final concentration of 1 % antibiotic in 50 mL of medium.
4. hWJC Culture Medium: Mix 445 mL DMEM containing low glucose and pyruvate with 50 mL of Fetal Bovine Serum (FBS) that is mesenchymal stem cell-qualified (MSC; Life Technologies). Store in aliquots of 25 mL at −20 °C. When ready to use, thaw aliquots O/N in 4 °C refrigerator, then heat-deactivate aliquots at 72 °C for 30 min the next day. Cool to 37 °C before use and add 5 mL penicillin-streptomycin (10,000 U/mL). Gently mix all components together by pipetting.
5. 0.05 % Trypsin-EDTA phenol red. Store aliquots of 25 mL at −20 °C, then thaw aliquots in 37 °C bead bath before use.

2.2 Freezing and Thawing hWJCs

1. hWJC Culture Medium (*see* Subheading 2.1, **step 4**).
2. Recovery™ Cell Culture Freezing Medium (Life Technologies).
3. 10 mM stock Y27632 Rho-associated coiled-coil kinase (ROCK) inhibitor (Reagents Direct, Encinitas, CA): Reconstitute 2 mg of inhibitor in 624 μL of Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich). Store in 104 μL aliquots in dark at −20 °C, until use.
4. hWJC Thawing Medium: Mix 500 μL of reconstituted Y27632 ROCK Inhibitor with 395 mL of DMEM by gently pipetting. Protect solution from light. Sterifilter DMEM and Y27632 ROCK inhibitor solution through a 500-mL EMD Millipore Stericup™ Sterile Vacuum Filter Unit. Add 100 mL of FBS-MSC-qualified (*see* Subheading 2.1, **step 4**) to DMEM to obtain a 20 % solution. Then add 5 mL of penicillin-streptomycin (10,000 U/mL) to DMEM to obtain a 1 % solution.
5. 0.05 % Trypsin-EDTA, phenol red (Life Technologies).
6. PBS (*see* Subheading 2.1, **step 1**).

2.3 Stem Cell Characterization

1. Primary Blocking Solution: Mix 0.6 g of 10 % BSA in 1 mL of Normal Donkey Serum (NDS). Use serum from secondary host. For example, if the secondary antibody label comes from a donkey host, then use normal donkey serum. Donkey, Rabbit, and Goat are common secondary hosts. Adjust volume to 10 mL with 1× PBS (*see* Subheading 2.1, **step 1**).

2. Secondary Blocking Solution: Mix 0.6 g of BSA in 10 mL of 1× PBS (*see* Subheading 2.1, **step 1**)
3. SYTOX® Red Dead Cell Stain (Life Technologies).
4. Isotype controls: Fluorescein isothiocyanate (FITC) IgG_1, Allophycocyanin (APC) IgG_1, R-Phycoerythrin (PE) IgG_1, Brilliant Violet (BV) IgG_1 (BD Biosciences, San Jose, CA).
5. Fluorescent secondary antibodies: Donkey Anti-Mouse Alexa Fluor 568 IgG, Donkey anti-mouse Qdot® 525 IgG (Life Technologies).
6. Primary antibodies: FITC mouse anti-human CD73, APC mouse anti-human CD90, PE mouse anti-human CD45, and BV421 mouse anti-human CD34 from BD Biosciences. Human STRO-1 antibody and human endoglin/CD105 antibody from R&D Systems (Minneapolis, MN).
7. hWJC Culture Medium (*see* Subheading 2.1, **step 4**).
8. 0.05 % Trypsin-EDTA, phenol red (Life Technologies).
9. PBS (*see* Subheading 2.1, **step 1**).

2.4 Transfection

1. P1 Primary Cell 4D-Nucleofector® X Kit (24 RCT) (Lonza Group Ltd, Basel, Switzerland).
2. Transfection Medium: Mix 500 μL of thawed 10 mM Y27632 ROCK Inhibitor (*see* Subheading 2.3) with 440 mL of Basal Medium from MSCGM™ Mesenchymal Stem Cell Growth Medium BulletKit via gentle pipetting. Sterifilter Basal Medium and Y27632 ROCK inhibitor through a 500-mL EMD Millipore Stericup™ Sterile Vacuum Filter Unit. Add remaining components of MSCGM™ Mesenchymal Stem Cell Growth Medium BulletKit to Basal Medium according to the manufacturer's instructions. Protect from light.
3. HATH1-CMV-plasmid (1 μg/μL): The HATH1 sequence (NCBI Reference Sequence: NC_000072.6) was sub-cloned into a PrecisionShuttle mammalian vector with N-terminal mGFP (Origene, Rockville, MD).
4. 0.05 % Trypsin-EDTA, phenol red (Life Technologies).
5. PBS (*see* Subheading 2.1, **step 1**).

2.5 Cell Staining

1. Dyes: Hoechst 33342 and FM® 1-43FX (Life Technologies).
2. 4 % Paraformaldehyde fixation solution: In a glass beaker on a stir plate in a well ventilated fume hood, Heat 800 mL of Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} to 60 °C. Add 40 g of paraformaldehyde (96 % extra pure) and then slowly add 1 mol/L NaOH drop-wise until the solution clears. Allow solution to cool to RT and adjust volume to 1 L with HBSS. Adjust pH to 6.9 by adding 1 mol/L HCl.

3. Mounting medium with DAPI.
4. Hank's Buffered Saline Solution (HBSS).
5. PBS (*see* Subheading 2.1, **step 1**).

2.6 Gene Expression Analysis

1. RNA purification kit (e.g., RNeasy; Qiagen, Valencia, CA).
2. Tissue homogenizer (e.g., QIAshredder; Qiagen).
3. 18 MΩ Milli-Q Water.
4. 100 % EtOH.
5. RNA ScreenTape system, including sample buffer and ladder (Agilent Technologies, Santa Clara, CA).
6. D1000 ScreenTape system, including reagents and ladder.
7. 10, 200, and 1000 μL aerosol barrier filter pipette tips.
8. cDNA reverse transcription kit.
9. DEPC-treated H_2O.
10. Primer-probe RNA detection kit (e.g., TaqMan® Fast Universal PCR Master Mix).
11. RNase decontamination reagent (e.g., RNase *AWAY*™).
12. 0.2-mL PCR tubes and caps, RNase-free.
13. 0.1-mL 96-well reaction plate with barcode (e.g., MicroAmp® Fast Optical; Life Technologies).
14. MicroAmp® optical adhesive film kit (Life Technologies).
15. TaqMan® Assays: *ATOH1*, *BARHL1*, *GAPDH, GFI1, HES1, HES5, JAG1, JAG2*, *MYO6, MYO7A, NOTCH1, POU4F3, SOX2* (Life Technologies).

2.7 Immunohistochemistry

1. 4 % Paraformaldehyde fixation solution in PBS: Heat 800 mL of PBS to 60 °C in a glass beaker on a stir plate in a well-ventilated fume hood. Continue as in Subheading 2.5, **step 2**, except use PBS.
2. PBS (*see* Subheading 2.1, **step 1**).
3. Detergents: Triton X-100, Tween 20.
4. Primary and Secondary Blocking Solutions (*see* Subheading 2.3, **steps 1** and **2**).
5. Histological mounting solution.
6. Secondary antibodies: Donkey anti-rabbit IgG (H + L), donkey anti-mouse IgG (H + L), donkey anti-goat IgG (H + L), Qdot® 525, 605 and 655 (Life Technologies).
7. Qnuclear™ Deep Red Stain (Life Technologies).
8. Primary antibodies: Rabbit anti-human Myosin VIIA (Novus Biologicals, Littleton, CO), mouse anti-human Neurog1 (Antibodies-Online, Atlanta, GA), goat anti-human Sox2 (R&D Systems).

2.8 Specialized Equipment and General Supplies

2.8.1 Equipment

1. Spinning disk confocal inverted microscope (e.g., Olympus IX81).
2. Inverted epifluorescence microscope (e.g., Olympus IX81).
3. Cell sorter.
4. Nucleofector (e.g., Lonza Group Ltd. 4D).
5. Thermo Scientific NanoDrop 2000.
6. Tape Station (e.g., Agilent Technologies 2200).
7. Thermocycler (e.g., Eppendorf Mastercycler®).
8. Instrument sterilizer (e.g., Lab Armor Bead Bath).
9. Biosafety cabinet.
10. Water-jacketed CO_2 incubator.
11. Centrifuge (e.g., Sorvall™ ST 40R) and microcentrifuge.
12. Smart tissue processor (e.g., Leica ASP300).
13. Paraffin embedding station (e.g., Leica EG1150C).
14. Microtome (e.g., Leica RM2255).
15. Vacuum pump.
16. Sterilizer (e.g., Primus PSS500).
17. Cryogenic storage unit (e.g., Thermo Scientific Locator™ 6 Plus Rack and Box System).

2.8.2 Supplies

1. NIH Style Neuro Punches (Fine Science Tools).
2. Scalpel handle #3 with scalpel blades #10, 11, and 12.
3. Fine Scissors and extra fine Graefe forceps.
4. Bright-Line™ hemacytometer.
5. Tubes and Bottles: 15 and 50-mL conical tubes, sterile 5-mL polypropylene tubes, 2-mL microcentrifuge tubes, 2-mL round-bottom cryogenic vials, and autoclavable Pyrex glass bottles (250-mL, 500-mL, 1000-mL).
6. Tissue culture filter flasks: TPP—T75, T150, T300 (MidSci).
7. 6-Well tissue culture-treated plate.
8. 100-mm mono plate (Fisher Scientific).
9. 35-mm sterile petri dish.
10. Pipettes: 2-mL aspirating pipettes, 25-mL serological pipettes.
11. Sterile 70-μm cell strainer (Biosciences).
12. Single Channel Auto-Pipettors (2-μL, 20-μL, 200-μL, 1000-μL) and pipette tips (10-μL, 200-μL, 1000-μL).
13. Mr. Frosty™ Freezing Container (Thermo Scientific).
14. Small and large Kimwipes.

3 Methods

3.1 Isolation of hWJCs from Umbilical Cord

1. Sterilize tissue culture hood for 30 min with UV light.
2. Turn on tissue culture hood blower and lift sash ~24 cm. Allow circulation in tissue culture hood to flow for 10 min before use.
3. Spray down all interior surfaces of tissue culture hood with 70 % EtOH (*see* **Note 1**).
4. Prepare tissue culture hood with drop cloth, 100-mm mono plates, tools (scalpels, forceps, scissors, etc.), and 50-mL conical tubes.
5. Prepare and sterifilter both Wash Solution and Digestion Medium (*see* Subheading 2.1, **steps 1–3**). Warm the Wash solution and Digestion medium in 37 °C bead bath (*see* **Note 2**).
6. After wiping down with EtOH, transfer specimen cup/container containing umbilical cord in sterile saline into the tissue culture hood.
7. Carefully, transfer umbilical cord from specimen cup/container to 100-mm mono plate. Remove ends of umbilical cords in 100-mm mono plate with scalpel. Section cord into ~3 cm increments. Squeeze out any residual or remaining blood from tissue sections with forceps.
8. Wash umbilical cord segments 2× with Washing Solution. Move cord segments around and aspirate off waste between. (It is imperative that as much blood is removed as possible.)
9. Transfer umbilical cord segments to new 100-mm mono plate.
10. Wash umbilical cord segments 2× with Washing Solution. Moving cord segments around and aspirate off waste between. (Remove all blood if possible.)
11. Transfer umbilical cord segments to a fresh 100-mm mono plate. Discard previous mono plates. Add just enough Wash Solution to keep umbilical cord segments moist.
12. Remove blood vessels from each umbilical cord segment (Fig. 1). Discard vessels (*see* **Notes 3** and **4**).
13. Wash umbilical cord segments twice with Wash Solution. Move umbilical cord segments around and aspirate waste between.
14. Transfer tissue segments to new 100-mm mono plate. Use scalpel to physically degrade tissue (*see* **Note 5**).
15. Once umbilical cord tissue is degraded to the consistency of "pulp," add Digestion Medium to the 100-mm mono plate via a pipette. Cut any floating large tissue pieces to smaller sizes (*see* **Note 6**).

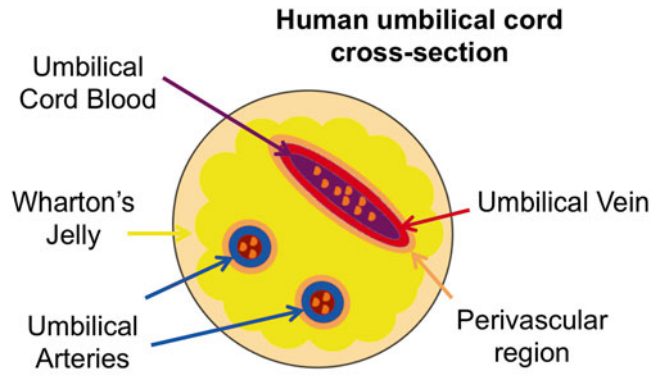

Fig. 1 Cross-section of human umbilical cord. The human umbilical cord contains three blood vessels that must be removed when harvesting human Wharton's jelly cells. Wharton's jelly is the gelatinous connective tissue found between the blood vessels in the human umbilical cord

16. Place the lid over the mono plate and place in a 37 °C incubator with 5 % CO_2 on an orbital shaker for a minimum of 4 h at 50 rpm (*see* **Note 7**).
17. Remove 100-mm mono plate from incubator. (If any large tissue pieces are still floating, place the mono plate back on the shaker in the incubator for 1 h.) Dilute digested tissue at a ratio of 1:4 with Washing Solution in 50-mL conical tubes.
18. Centrifuge conical tubes for 10 min at ~500 × *g*. Check for pellet. If no pellet is visible, dilute with additional Washing Solution to a ratio of 1:8 and centrifuge. A dilution of 1:16 is sometimes necessary for a pellet to form.
19. Aspirate supernatant from tubes. Resuspend pellet in hWJC Medium.
20. Count cells using a hemocytometer.
21. Plate 7000 cells/cm^2 in a T-flask.
22. Gently wash cells with sterile PBS and replace medium every 6–8 h in the first 24 h following plating, to remove residual blood cells.
23. Replace medium every 48 h and sub-culture cells when culture vessel reaches 80 % confluence.

3.2 Culturing and Sub-culturing hWJCs

1. Place hWJC Culture Medium in 37 °C bead bath for 30–40 min (*see* **Note 8**).
2. Prepare tissue culture hood as in Subheading 3.1, **steps 1–3**.

3.2.1 Culturing hWJCs

3. Turn on vacuum pump, and spray inside and outside of tubing.
4. Spray and wipe down all reagents and tools with 70 % EtOH before placing in tissue culture hood (e.g., pipettors, culture medium, conical tubes, etc.) (*see* **Note 9**).

Table 1
Medium recommendations for well plates

Well-plate format	Medium volume to add per well (mL)
96-Well plate	0.2
48-Well plate	0.5
24-Well plate	1
12-Well plate	1.5
6-Well plate	3

Table 2
Medium recommendations for T-flasks

T-flask size	Medium volume to add (mL)
T-25 cm^2	5
T-75 cm^2	15
T-150 cm^2	25
T-300 cm^2	30

5. Gently clean stage of inverted light microscope with 70 % EtOH.
6. Remove cell culture vessel from 37 °C CO_2 incubator.
7. Check cell confluence and health using an inverted light microscope (*see* **Note 10**).
8. Gently wipe outside of cell culture vessel with 70 % EtOH and place in tissue culture hood.
9. Open wide end of aspirating pipette and hook up to aspirating tubing.
10. Remove pipette wrapper, and carefully remove well plate cover or T-flask cap.
11. Aspirate out all medium, but *do not* touch cell surface with aspirating pipette (*see* **Notes 11** and **12**).
12. After removing medium, *carefully and slowly* add fresh warm medium to cell culture vessel (Tables 1 and 2) (*see* **Notes 13** and **14**).
13. Replace lid or cap on culture vessel and return to 37 °C CO_2 incubator (*see* **Note 15**).

3.2.2 Sub-culturing hWJCs (Passaging)

1. Aspirate all medium off of cells.
2. Wash cells by adding sterile PBS to cells in an amount equivalent to the cell culture medium.
3. Rock T-flask or well plate back and forth gently to spread PBS over entire cell-covered surface, then aspirate all PBS off of cells.
4. Repeat **steps 2** and **3**, Subheading 3.2.2 one more time.
5. Add 0.05 % Trypsin-EDTA to cells in an amount equivalent to the cell culture medium.
6. Rock T-flask or well plate back and forth briefly to spread 0.05 % Trypsin-EDTA over the entire cell-covered surface.
7. Place T-flask or well plate in 37 °C incubator for 3–5 min.
8. While T-flask or well plate is in incubator, label and prepare 50-mL conical tube(s) under the tissue culture hood with an amount of growth medium that is equivalent to the 0.05 % Trypsin-EDTA used to detach cells. For example, if 2 mL of 0.05 % Trypsin-EDTA was added to each well of a 6-well plate, then add 2 mL of growth medium to an empty 50-mL conical tube under the tissue culture hood.
9. Prepare a separate conical tube for each well or flask in which cells are being detached. Label all conical tubes accordingly to keep track of source for lifted cells.
10. Remove T-flask or well plate from incubator and verify that cells have detached from the surface using a light-inverted microscope. If 90 % ≤ cells have detached, gently tap side of flask or well plate, to enhance cell detachment, and verify detachment using microscope.
11. Place T-flask or well plate underneath the tissue culture hood and transfer 0.05 % Trypsin-EDTA into designated conical tube containing equivalent amount of growth medium. The ratio of growth medium to 0.05 % Trypsin-EDTA should be 1:1, to deactivate the enzyme.
12. Pellet the cell suspension down by spinning the conical tube in the centrifuge at ~500 × *g* for 5 min.
13. Aspirate off all 0.05 % Trypsin-EDTA/hWJC Medium solution (*see* **Note 16**) and resuspend cells in 1 mL of hWJC Medium.
14. Count cells using a hemocytometer or automated cell counter.
15. Once total cell number is determined, dilute cells accordingly and split into new T-flasks or wells. hWJCs grow most efficiently at a density of 7000 cells/cm^3.

3.3 Freezing and Thawing hWJCs

3.3.1 Freezing Cells

1. Pre-label 2-mL cryogenic vials with threaded caps denoting: date, cell type, name, and passage number. Reference notation to umbilical cord (internal numbering of deidentified sample).
2. Follow sub-culture protocol (*see* Subheading 3.2.1, **steps 1–14**).
3. Make note of how many cells are in 1 mL of medium. Repellet cell suspension, by centrifuging at ~500 × *g* for 5 min.
4. Aspirate supernatant and resuspend cells in Recovery™ Cell Culture Freezing Medium (*see* Subheading 2.2, **step 2**) at a concentration of 1×10^6 cells/mL. Work quickly as this medium contains Dimethyl sulfoxide (DMSO).
5. Aliquot 1 mL of cell suspension into each pre-labeled cryogenic vial and cap vials.
6. Transfer vials into a Mr. Frosty™ Freezing container filled with isopropanol and place container into a −80 °C freezer for up to 12 h.
7. Remove container from −80 °C freezer and quickly transfer cryogenic vials into a liquid nitrogen-filled cryopreservation storage unit.
8. Store samples until ready for use.

3.3.2 Thawing Cells

1. Warm hWJC Thawing Medium (*see* Subheading 2.3, **step 4**) and 50 mL of sterile PBS to 37 °C.
2. Place a 35-mm petri dish and appropriate number of T-75 flasks under the tissue culture hood, and label accordingly. Use two T-75 flasks per vial.
3. After hWJC Thawing Medium and PBS are warm, place under tissue culture hood.
4. Fill a 50-mL conical tube with 49 mL of pre-warmed thawing medium.
5. Fill 35-mm petri dish with warm PBS.
6. Quickly retrieve frozen vials of cells from cryopreservation storage unit and spray vials with 70 % EtOH, then place under tissue culture hood.
7. Place bottom of cryogenic vials in warm PBS (*see* **Note 17**).
8. Draw up ~0.5 mL of hWJC Thawing Medium from the 50 mL conical tube, using the 2-mL aspirating pipette.
9. Carefully pipette hWJC Thawing Medium into cryogenic vial containing frozen cells. *Do not take vial out of PBS.* Slowly transfer cell solution and hWJC Thawing Medium between cryogenic vial and 50 mL conical tube until vial is empty of cell solution.
10. Gently mix cells in hWJC Thawing Medium by gently pipetting up and down, or gently rocking closed 50-mL conical tube back and forth.

11. Aliquot 25 mL of cell suspension into each pre-labeled T-75 flask (2/cryogenic vial).
12. Lay T-75 flask on side and swirl medium and cells around to evenly distribute them across the bottom, then place flasks in 37 °C incubator.
13. Repeat **steps 10–12,** Subheading 3.3.2 for each additional vial of cells.
14. After 24 h, replace hWJC Thawing Medium with hWJC Culture Medium and culture hWJCs according to hWJC culture protocol described in **steps 1–13**, Subheading 3.2.1.

3.4 Stem Cell Characterization

It is highly recommended that 1.6×10^7 or more cells are used for this protocol to ensure enough cells are available for flow cytometry analysis.

1. Pre-label 16 2-mL microcentrifuge tubes and 16 5-mL polypropylene tubes for cell characterization as follows:
   - One tube for cells that will not be stained and are used for baseline measurements.
   - One tube for each CD marker (6 tubes total).
   - One tube for each isotype control (6 controls total).
   - Three tubes with cells stained for all CD markers.
2. Follow sub-culture protocol (*see* Subheading 3.2.2, **steps 1–15**).
3. Make note of how many cells are in 1 mL of medium. Repellet cell suspension by centrifuging at ~500 × *g* for 5 min.
4. Aspirate off supernatant and resuspend cells in Primary Blocking Solution (*see* Subheading 2.3, **step 1**) at a concentration of 1×10^6 cells/mL. Aliquot 1×10^6 cells/mL cells into pre-labeled 2-mL microcentrifuge tubes. Place cells on ice and keep in the dark for 20 min.
5. Microcentrifuge samples at ~1556 × *g* for 5 min at 4 °C, then decant supernatant.
6. Add appropriate amount of cell marker or isotype (Table 3) and corresponding amount of Secondary Blocking Solution (*see* Subheading 2.3, **step 2**), so that cells in each microcentrifuge tube are suspended in a total volume of 200 μL. Mix each suspension well with gentle pipetting (*see* **Note 18**).
7. Incubate cells on ice, and keep in dark for 20 min.
8. Add 800 μL of Secondary Blocking Solution to each centrifuge tube to bring the total volume to 1000 μL.
9. Centrifuge samples at ~1556 × *g* for 5 min at 4 °C.
10. Decant supernatant.

Table 3
Cell marker volumes

CD marker/isotype	Recommended marker volume (μL)	Cell number	2° Blocking solution (μL)
Unstained	0	1,000,000	200
Mouse anti-human STRO-1 (1°)	2.5	1,000,000	197.5
Donkey anti-mouse Alexa Fluor 568 (2°)	2	1,000,000	198
Mouse anti-human human CD105 (1°)	2.5	1,000,000	197.5
Donkey anti-mouse Qdot 525 (2°)	2	1,000,000	198
CD45 PE	5	1,000,000	198
PE isotype control	5	1,000,000	198
Mouse anti-human FITC CD73	5	1,000,000	195
FITC isotype control	5	1,000,000	195
Mouse anti-human brilliant violet CD34	5	1,000,000	195
Brilliant violet isotype	5	1,000,000	195
Mouse anti-human APC CD90	5	1,000,000	195
APC isotype control	5	1,000,000	195

11. Add appropriate amount of secondary antibody-fluorophore and Secondary Blocking Solution to samples that require secondary labeling as prescribed in Table 3 (*see* **Note 19**). Cells that do not require secondary labeling should be suspended in 1000 μL of Secondary Blocking Solution.
12. Incubate cells on ice and keep in dark for 20 min.
13. Add 800 μL of Secondary Blocking Solution to secondary labeled cells.
14. Centrifuge secondary labeled samples at ~1556 × *g* for 5 min at 4 °C.
15. Decant supernatant.
16. Suspend cells that do not require further labeling in 1000 μL of Secondary Blocking Solution and place on ice. Continue sequential labeling of cell markers for samples to be stained with all cell markers by repeating **steps 7–17**, Subheading 3.4, until designated samples are labeled for all markers.
17. After all samples are labeled, centrifuge cells at ~1556 × *g* for 5 min at 4 °C.
18. Decant supernatant.
19. Resuspend all samples in 400 μL of PBS.

20. Carefully strain each cell suspension through a 70-μm nylon cell strainer into its respective polypropylene tube to break up cell aggregates.
21. Add optional dyes to cell suspensions if desired (e.g., Hoechst, SYTOX® Red Dead Stain).
22. Analyze cell samples with Beckman Coulter MoFlo XDP cell sorter (*see* **Notes 20** and **21**).

3.5 Transfection

1. Culture hWJCs, used for transfection experiments, with medium components provided in the Lonza BulletKit. Medium provided in BulletKit is mixed and handled according to the manufacturer's instructions. hWJCs are cultured to 80 % confluency before beginning transfection protocol.
2. Warm PBS, Transfection Medium, and Trypsin to 37 °C 2 h prior to transfection.
3. Remove medium from hWJCs, and gently wash cells 2× with PBS, 1 h prior to transfection. After removing PBS, replenish cell cultures with Transfection Medium (*see* Subheading 2.4, **step 2**) and place in a 37 °C CO_2 incubator for 1 h.
4. While hWJCs are incubating, place HATH1 plasmid DNA on ice. If necessary, reconstitute fresh 4D Nucleofector P1 Primary cell solution, according to the manufacturer's instructions, and refrigerated at 4 °C. Label 100-μL 4D-Nucleofector cuvettes accordingly. Label 6-well plates, fill with 2.4 mL of Transfection Medium and place in a 37 °C cell culture grade incubator.
5. After 1 h, detach hWJCs according to Sub-culture procedure (*see* Subheading 3.2.2, **steps 1–14**).
6. After counting cells, repellent cell suspension by centrifuging at ~500 × *g* for 5 min.
7. Resuspend cells in 4D-Nucleofector P1 Primary cell solution at a concentration of 5×10^6 cells/1 mL. Separate cells corresponding to experimental parameters (e.g., untreated controls, HATH1-transfected cells, any other designated treatment groups). Plate non-transfected cells immediately into designated wells containing transfection medium. Pipette 100 μL of cell suspension into each well designated as an untreated control.
8. Add appropriate amount of HATH1 plasmid DNA (1 μg/μL) to cells designated for transfection.
    - Up to 5 μg of HATH1 plasmid DNA per 5×10^5 cells can be added for efficient transfection. Depending on experiment parameters between 2 and 3 μg of HATH1 plasmid DNA per 5×10^5 works well for most applications.
    - The volume of plasmid DNA should not exceed 10 % of total volume of cell suspension.

9. Gently mix DNA with cell suspension by tapping tube containing cells.
10. Aliquot 100 μL of DNA/cell suspension into 100-μL 4D Nucleofector cuvettes. After transferring cell suspension into cuvettes, gently tap cuvettes twice to evenly distribute cell suspension between electrodes.
11. Transfer cuvettes to 4D Nucleofector X-unit. Select Human MSC cell type and program FF-104 on the touch screen interface of the 4D Nucleofector Core-unit. Follow software prompts, and Nucleofect cells.
12. After Nucleofection, allow cuvettes to incubate at RT for 10 min.
13. Using the transfer pipette provided with the 4D Nucleofector MSC kit, transfer 100 μL of cells from each cuvette to the designated well in a 6-well plate. Gently swirl plate to evenly distribute cells.
14. Place 6-well plates in 37 °C CO_2 incubator.
15. After 24 h, replace transfection medium with Lonza MSC BulletKit Medium.
16. Culture cells and analyze according to experimental parameters.

3.6 Cell Staining

All cells designated for staining are sub-cultured and plated on a fibronectin-coated glass cover slip. Culture cover slips in 6-well plates until ready to stain.

3.6.1 Hoechst Staining

1. Remove medium, and wash 2× with HBSS, then add 1 mL of HBSS to each well.
2. Add 0.5 μL of Hoechst 33342 to each well and incubate cells at 37 °C in an incubator for 10 min.
3. Image cells using an inverted epifluorescent microscope (e.g., Olympus IX81) or a spinning disk confocal inverted microscope (e.g., Olympus IX81) and use the guide parameters in Table 4 to view and image labels.

3.6.2 FM 1-43 FX

1. Prepare a working staining solution of 5 μg/mL of FM 1-43 FX in ice-cold HBSS. Keep solution on ice.
2. Remove medium from cover slip, and immerse cover slip in FM 1-43 working staining solution on ice for 1 min.
3. Remove cover slip from FM 1-43 FX working staining solution and immerse with ice-cold 4 % paraformaldehyde in HBSS for 10 min to fix cells. Keep cover slip and 4% paraformaldehyde on ice.
4. Rinse the cover slip at least three times with HBSS.

Table 4
Excitation and emission parameters

Marker	Microscope	Light source	Excitation (nm)	Emission (nm)
Hoechst 33342	IX81 Inverted Epi	Mercury Arc Lamp	387 ± 11	446 ± 60
GFP	IX81 Inverted Epi	Mercury Arc Lamp	494 ± 20	531 ± 22
Alexa Fluor 555	IX81 Inverted Epi	Mercury Arc Lamp	575 ± 25	624 ± 40
Alexa Fluor 647	IX81 Inverted Epi	Mercury Arc Lamp	650 ± 25	684 ± 25
FM 1-43 FX	IX81 Inverted Confocal	405 nm solid-state laser	405	625 ± 11
Qdot 525	IX81 Inverted Confocal	405 nm solid-state laser	405	531 ± 22
Qdot 565	IX81 Inverted Confocal	405 nm solid-state laser	405	560 ± 25
Qdot 605	IX81 Inverted Confocal	405 nm solid-state laser	405	613 ± 20
Qdot 655	IX81 Inverted Confocal	405 nm solid-state laser	405	655 ± 15
Qdot 800	IX81 Inverted Confocal	405 nm solid-state laser	405	788 ± 20
Qnuclear™ Deep Red Stain	IX81 Inverted Confocal	642 nm solid-state laser	642	655 ± 15

GFP green fluorescent protein

5. Follow manufacturer's instructions, and mount and seal cover slip with ProLong® Gold Antifade Reagent with DAPI.
6. Image cells (Fig. 2) as in Subheading 3.6.1, **step 3**.

3.7 Gene Expression Analysis

3.7.1 RNA Isolation

1. Remove medium from cells, and wash 2× with PBS.
2. Harvest RNA using RNeasy Plus Mini isolation kit following manufacturer's instructions (*see* Subheading 2.6, **step 1**).
3. RNA may be stored and preserved at −80 °C for later use, if not immediately converted to cDNA. Avoid multiple freeze–thaw cycles.

3.7.2 NanoDrop 2000 RNA Analysis

Perform **steps 1–5**, Subheading 3.7.2, before starting NanoDrop 2000 RNA analysis.

1. Set bead bath to 72 °C.
2. If necessary, thaw RNA on ice.
3. Remove Agilent Technologies RNA ScreenTape and RNA ScreenTape buffer from refrigerator, and bring to RT in ~30 min.
4. Pipette 5 μL of DEPC-treated water (*see* Subheading 2.6, **step 9**) on the NanoDrop 2000 quartz crystal pedestal and lower the arm. Allow DEPC-treated water to incubate for 5 min to clean quartz crystal and then remove water with a Kimwipe.

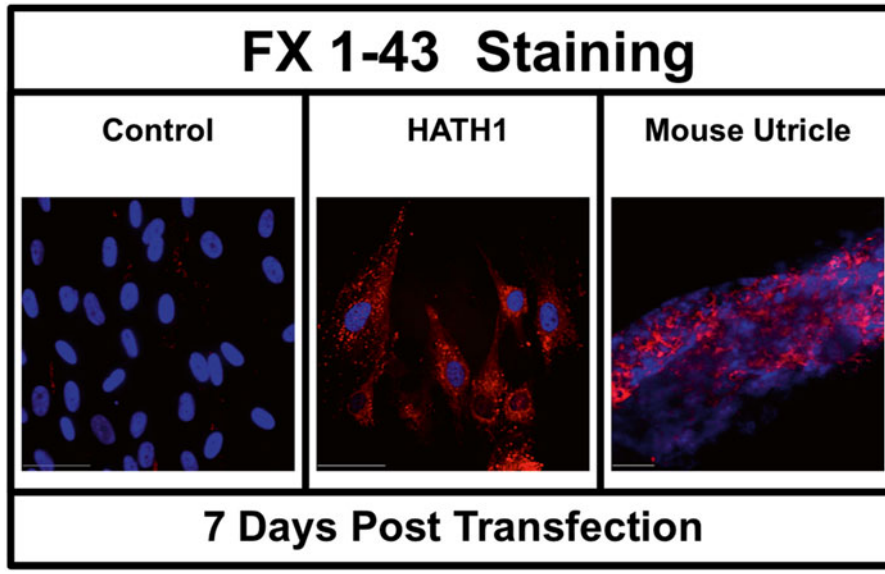

Fig. 2 FM 1-43 staining of lipid membranes. FM 1-43 is a lipophilic dye that is used to identify transfection channels found in mechanosensory hair cells. Sensory epithelium containing hair cells found in the mouse utricle and HATH1-transfected hWJCs revealed positive infiltration of FM 1-43 (*Red*) into cells. Cell nuclei were stained with DAPI (*Blue*). Scale Bars = 50 μm

5. Turn on Agilent TapeStation.
6. Once **steps 1–5**, Subheading 3.7.2 are completed, open NanoDrop 2000/2000c software and close NanoDrop 2000 arm, if not already closed.
7. Select "Nucleic Acids" Button in software.
8. Select RNA in the drop-down menu in the upper right corner.
9. Raise NanoDrop 2000 arm, pipette 1 μL of RNase-Free water on the quartz crystal, and close NanoDrop 2000 arm carefully.
10. Hit "Blank" button to measure background of RNase-Free water. Be sure to use the same solution that is suspending your RNA sample as the Blank, to get an accurate background reading.
11. When the "Measure" button becomes selectable, raise the NanoDrop 2000 arm and wipe off the quartz crystal carefully with a Kimwipe. Pipette 1 μL of RNA sample onto the quartz crystal, and carefully close the NanoDrop 2000 arm. In the upper right corner of the screen, name the sample being tested. Then select "Measure" (*see* **Notes 22** and **23**).
12. After measuring is complete, carefully raise the NanoDrop 2000 arm, and wipe off the quartz crystal with a Kimwipe. Load 1 μL of RNA from the next sample and lower the NanoDrop 2000 arm. Label the sample accordingly in the software and measure.

13. Repeat **step 7**, Subheading 3.7.2 for each new sample. Wipe off quartz crystal every time after measuring a sample.
14. After measuring all samples, create a report to export all the information. Wipe off the quartz crystal, load 5 μL of DEPC-treated water on the quartz crystal and lower the NanoDrop 2000 arm to clean. Allow the DEPC-treated water to spread over the crystal for 5 min, then wipe off the quartz crystal with a Kimwipe.

3.7.3 Agilent Technologies 2200 TapeStation RNA Analysis

1. Start up Agilent TapeStation software, but *not* the Agilent Analyzer Software.
2. Briefly vortex RNA ScreenTape Reagents.
3. Briefly centrifuge RNA ScreenTape Reagents.
4. Load RNA ScreenTape into TapeStation.
5. Label up to 16 optical tubes (2 8-tube strips) on the side accordingly.
6. Add 4 μL of R6K reagent buffer to each tube.
7. Add 1 μL of RNA sample to each tube.
8. Denature RNA samples by heating samples in a 72 °C water bath for 3 min. Immediately place RNA samples on ice and incubate for 2 min.
9. Briefly centrifuge RNA samples to collect contents.
10. Load tubes into TapeStation and remove tube-strip caps. *It is imperative to remove tube-strip caps, otherwise the internal TapeStation automated pipettor will break.*
11. Load appropriate number of pipette tips into TapeStation.
12. In software, label tubes to be analyzed.
13. Close TapeStation Lid and save RNA file, then click "START" program.
14. After running the program, there should be clear bands in the gel image. The peaks should also be sharp and well-defined in the electropherogram. The RNA Integrity Number (RIN) should be between 7 and 10. Do not use any RNA with a RIN below 7. Any RNA with a RIN below 7 is too far degraded to be reliable in downstream applications.

3.7.4 RNA to cDNA Conversion

1. Before starting, thaw High Capacity cDNA reverse transcription kit components (*see* Subheading 2.6, **step 8**), then mix Master Mix according to the manufacturer's instructions.
2. Pipette 10 μL of 2× RT Master Mix into each well of a 96-well reaction plate or individual tube.
3. Pipette 10 μL of RNA sample into each well, pipetting up and down 2× to mix.

4. Seal the plates or tubes.
5. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
6. Place the plate or tubes on ice until ready to load the thermal cycler.
7. Program thermal cycler using the following parameters: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, hold at 4 °C until ready to remove.

3.7.5 NanoDrop 2000 cDNA Analysis

1. Thaw DNA on ice.
2. Pipette 5 μL of DEPC-treated water onto the NanoDrop 2000 quartz crystal pedestal and lower NanoDrop 2000 arm. Allow DEPC-treated water to incubate for 5 min to clean quartz crystal, then remove water with a Kimwipe.
3. Open NanoDrop 2000/2000c software and close NanoDrop Arm, if not already closed.
4. Select "Nucleic Acids" button.
5. Select DNA in the drop-down menu in the upper right corner.
6. Raise NanoDrop 2000 arm, and pipette 1 μL of DEPC-treated water onto the quartz crystal and close NanoDrop 2000 arm carefully.
7. Hit "Blank" button to measure background of RNase-Free water. Be sure to use the same solution that is suspending your cDNA sample in the Blank, to get an accurate background reading.
8. When the "Measure" button becomes selectable, raise the NanoDrop 2000 arm and wipe off the quartz crystal carefully with a Kimwipe. Pipette 1 μL of cDNA sample onto the quartz crystal and carefully close the NanoDrop 2000 arm. In the upper right corner of the screen, name the sample being tested. Then select "Measure" (*see* **Note 24**).
9. Carefully, raise the NanoDrop 2000 arm and wipe off the quartz crystal with a Kimwipe. Load 1 μL of cDNA from the next sample and lower the NanoDrop 2000 arm. Label the sample accordingly in the software and measure.
10. Repeat **step 7** for each new sample. Wipe off quartz crystal every time after measuring a sample.

3.7.6 cDNA Gene Expression Analysis via RT-qPCR

1. Turn On Eppendorf Realplex and computer, open Realplex software and login with appropriate user account.
2. Fill a cooler with ice. Remove Universal Fast Master Mix (*see* Subheading 2.6, **step 10**) from refrigerator and place contents on ice.

Table 5
TaqMan primers used for RT-qPCR

Gene name	Gene symbol	Species	Dye	Entrez gene ID	Life technologies ID
Atonal homolog 1	ATOH1	Human	Fam	474	Hs00944192_s1
BarH-like homeobox 1	BARHL1	Human	Fam	56751	Hs01063929_m1
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Human	Fam	2597	Hs02758991_g1
Growth factor independent 1 transcription repressor	GFI1	Human	Fam	2672	Hs00382207_m1
HES family bHLH transcription factor 1	HES1	Human	Fam	3280	Hs00172878_m1
HES family bHLH transcription factor 5	HES5	Human	Fam	388585	Hs01387463_g1
jagged 1	JAG1	Human	Fam	182	Hs01070032_m1
jagged 2	JAG2	Human	Fam	3714	Hs00171432_m1
Myosin VI	MYO6	Human	Fam	4646	Hs01568216_m1
Myosin VIIA	MYO7A	Human	Fam	4647	Hs00934542_m1
Notch 1	NOTCH1	Human	Fam	4851	Hs01062014_m1
POU class 4 homeobox 3	POU4F3	Human	Fam	5459	Hs00231275_m1
SRY (sex determining region Y)-box 2	SOX2	Human	Fam	6657	Hs01053049_s1

3. Remove cDNA samples and TaqMan Assays (*see* Subheading 2.6, **step 15**) from −20 °C freezer and thaw on ice (Table 5).
4. Spray off gloves and countertop with RNase Away (*see* Subheading 2.6, **step 12**).
5. Program "Plate Layout" within Realplex software.
6. Program the RT-qPCR Cycle, following the manufacturer's instructions.
7. Add 10 μL of Master Mix (2×) to each well.
8. Add 1 μL of TaqMan Assay to each well. Remember to change tips after pipetting TaqMan Assay into each well.
9. Add 9 μL of sample to each well. Remember to change tips after pipetting sample into each well. Mix reagents and samples directly in 96-well plate by pipetting up and down 2×.
10. Seal plate with optical film. Use applicator to seal film tightly, but be careful not to bump the plate and cause liquid to splatter onto optical film.

11. Run the plate within 2 h, otherwise place the plate in a 4 °C refrigerator for short-term storage or in the −20 °C for long-term storage.
12. Run the RT-qPCR program and save file.
13. Use the ΔΔCt method to calculate changes in gene expression.
14. Run gel electrophoresis on samples and control DNA to verify fragment size for further verification of DNA amplification. May be executed with 2200 TapeStation and D1000 Screen Tape and Reagents, according to the manufacturer's instructions.

3.8 Immunohistochemistry

Cells should be grown on tissue-coated glass cover slips for this process.

3.8.1 Processing Cells

1. Remove medium, and wash cover slips with PBS 2×.
2. Immerse cover slips in 4 % Paraformaldehyde fixation solution (*see* Subheading 2.7, **step 1**) for 15 min.
3. Wash cover slips with PBS 3× for 5 min each.
4. Immerse cover slips in 0.25 % Triton X-100 in PBS for 15 min to permeabilize cells at RT.
5. Wash cover slips with PBS 3× for 5 min each.
6. Proceed with immunostaining immediately.

3.8.2 Processing Tissue Constructs

1. Remove medium, and wash tissue construct with PBS 2× for 15 min each.
2. Immerse tissue construct in 4 % Paraformaldehyde fixation solution (*see* Subheading 2.7, **step 1**) in a volume 10×> the volume of the sample for 12–16 h (e.g., if your sample has a volume of 1 cm^3, immerse the sample in 10 mL of 4 % Paraformaldehyde fixation solution).
3. Using a Leica ASP300 smart tissue processor, use the following protocol to process tissues: Rinse tissue construct in tap water for 1 h, then wash in a graded increase of EtOH for 2 h each (30 %, 50 %, 70 %, 80 %, 90 %, 95 %), followed by three 1 h washes in 100 % EtOH and two 1 h washes in 100 % xylene. Then, immerse tissue construct 3× in fresh paraffin for 1 h each and embed tissue construct in paraffin.
4. Section paraffin-embedded tissue construct in 10 μm sections on a microtome and float onto a 40 °C water bath containing distilled water.
5. Carefully transfer the sections onto glass slides suitable for immunohistochemistry.
6. Place slides in a metal slide rack at a 45° angle inside an oven and incubate for 20–40 min at 65 °C to allow tissues to release moisture and bond to slide.

Table 6
Antibodies and labels

Primary antibody	Primary dilution	Secondary antibody	Secondary dilution
Rabbit anti-human MYOSIN VIIA	1:100	Donkey anti-rabbit Qdot 525	2:100
Mouse anti-human NEUROG1	1:100	Donkey anti-mouse Qdot 605	2:100
Goat anti-human SOX2	1:100	Donkey anti-mouse Qdot 655	2:100

7. Remove from oven and allow tissues to dry O/N at RT (*see* **Note 25**).
8. Deparaffinize and rehydrate tissues by submerging tissue for 3 min each in the following solutions: 100 % Xylene, 50 % xylene/ethanol (v/v), a graded decrease in ethanol (100 %, 95 %, 70 %, 50 %), followed by rinsing 2× in PBS for 5 min each. Once rehydrated, rinse tissue sections in 0.2 % Tween 20 in PBS 2× for 10 min each.
9. Wash tissue sections in PBS 2× for 5 min each.

3.8.3 Immunostaining

1. Block samples with Primary Blocking Solution (*see* Subheading 2.3, **step 1**) for 1 h.
2. Incubate samples with primary antibody in appropriate dilution of Secondary Blocking Solution (*see* Subheading 2.3, **step 2**) O/N at 4 °C (Table 6).
3. Wash samples with PBS 3× for 5 min each.
4. Incubate samples with secondary antibody label in appropriate dilution of Secondary Blocking Solution O/N at 4 °C (*see* Table 6) (*see* **Note 26**).
5. Wash samples with PBS 3× times 5 min each.
6. Counterstain samples with Qnuclear™ Deep Red Stain (*see* Subheading 2.8), according to the manufacturer's instructions.
7. Wash samples with PBS 3× 5 min each.
8. Dehydrate samples according to the following protocol (EtOH is diluted in ddH_2O): Submerge samples in increasing concentrations of EtOH for 5 s each: 30 %, 50 %, 70 %, 90 %, followed by submersion 2× in 100 % for 5 s each, and finally 2× 100 % xylene for 5 s each.
9. Immediately mount with mounting medium.
10. Image samples as in Subheading 3.6.1, **step 3** (Table 4).

4 Notes

1. Spray gloved hands with 70 % ethanol each time before placing in tissue culture hood.
2. Measure the umbilical cord length to the nearest centimeter. Prepare 50 mL of Digestion medium (*see* Subheading 2.1, **step 3**) for every 10 cm of umbilical cord. For example, for a 20 cm umbilical cord, prepare 100 mL of Digestion Medium. Do not allow umbilical cord tissues to dry out during isolation process. Always keep tissues moist with Washing Solution.
3. There are two arteries and one vein. The two arteries are typically 2–3 mm in diameter and look like "rubbery" white fibers. The vein looks like a large flattened "tube" with a thin tissue wall. The diameter of the vein may be twice the size of the arteries.
4. There are two ways to remove the blood vessels:
   - Method 1: Use two pairs of forceps. Use one pair of forceps to grab the end of a blood vessel and pull. Use the other pair of forceps to gently "peel" away the surrounding tissue. The arteries tend to pull out of the umbilical cord segment smoothly. The vein does not detach easily.
   - Method 2: Carefully, use the scalpel to make a length-wise incision through the amniotic epithelium to allow the umbilical cord segment to "unroll" from a tube to a flat piece of tissue. Use the forceps to gently pull the blood vessels away the umbilical cord segment. CAUTION: *When using this method, be carful not cut through a blood vessel, or the blood vessel will be difficult to identify and remove. Locate blood vessels before cutting into umbilical cord segment, and make incision away from blood vessels.*
5. Mince up the tissue until it has a "pulp-like" consistency.
6. For every 10 cm of umbilical cord, divide minced tissue into separate 100-mm mono plates for digestion. Add 50 mL of Digestion medium to each 100-mm mono plate. For example, if an umbilical cord is initially 30 cm in length, divide minced up tissue equally between 3 100-mm mono plates, and add 50 mL of Digestion medium to each 100-mm mono plate.
7. The length of the umbilical cord and physical degradation affect the amount of time needed to digest the umbilical cord tissue. The digestion process is more efficient when umbilical cord tissue is physically degraded into fine pieces than medium to large pieces. Digestion can take between 4 and 8 h, depending on the quantity of umbilical cord tissue processed and how well umbilical cord tissue was physically degraded.

8. If there is potential for a tube cap or bottle neck to come in contact with water, seal tube cap or bottle neck with parafilm.
9. When removing reagents from bead bath, they should feel warm to the touch, but not hot. If reagents feel cold, continue warming in the bead bath.
10. Cells should appear either spindle-shaped ("Crescent Moon") at low confluence, and fibroblastic ("Webbed") when over 50 % confluence. In addition, cells should have well defined edges and nuclei. Cells *should not* display signs of "blebbing."
11. For well plates, tilt plate at a 30°–45° so that medium is up against the well wall. Then aspirate medium from the wall without touching cell surface (floor of the well).
12. For T-flasks, gently turn flask over so that medium moves from the floor of the T-flask (cell culture surface) to the ceiling of the T-flask. Carefully insert the aspirating pipette into the T-flask without touching the edge or inside of the neck of T-flask and aspirate medium from the ceiling of the T-flask.
13. When adding medium to a well plate, use a pipette and aim for the wall of the well. *Do not* add medium directly to the cell surface (floor of the well), as cells may detach. Add medium slowly, so as not to disrupt the cell monolayer. Sometimes, it is helpful to aliquot medium from the main stock solution into a smaller container, when feeding cells in well plates, to avoid potentially contaminating the main stock solution of medium. For example, if the main stock solution of medium is in a 500-mL bottle and medium needs to be changed in three different 6-well plates, then pipette 54 mL of medium from the main stock into two 50-mL conical tubes. This way, medium is pipetted directly out of the conical tube, instead of the main stock.
14. When adding medium to a T-flask, position the T-flask so that it is nearly vertical and gently add medium to the ceiling of T-flask. Then, gently turn the T-flask over and lay down so that the medium covers the cell surface (floor of T-flask). *Do not* add medium directly to the cell surface.
15. Special Notes regarding cell culture:
    - Check cells and feed on a regular schedule, such as every 48 h.
    - Check cells before feeding. If cells have reached a confluency of 80–90 %, then cells need to be sub-cultured, that is, passaged (*see* Subheading 3.2.2).
    - Confluency—Coverage of cell surface area. For example, 50 % confluency means that cells cover 50 % of the surface area of the cell culture vessel.

- If black dots are observed that move against the motion of the liquid or if "segmented arms" are observed branching out across the surface area of the cell culture vessel, then cells are most likely contaminated with bacteria or fungi, respectively. Contaminated cell cultures will need to be disposed of according to state and local regulations of the institution.

16. Keep aspirating tip near mouth of tube and tilt tube to direct liquid toward aspirating tip. *Do not* place aspirating tip near cell pellet inside of tube.
17. Make sure the tip of the vial is submerged in PBS so that the vial does not explode.
18. Samples being labeled for all CD markers (CD34, CD45, CD73, CD90, CD105, STRO-1) will need to be sequentially labeled, to avoid cross-reactivity. One antibody or label can be applied at every incubation-wash step. An incubation-wash step consists of incubating a cell sample with an antibody or label for 20 min on ice in the dark. Afterward, the cell sample is washed with Secondary Blocking Solution and centrifuged to pellet the sample. The supernatant is decanted and the next incubation-wash step started. It is recommended that the STRO-1 primary antibody be added first, followed by the secondary donkey anti-mouse Alexa Fluor 568 label. Afterward, the CD105 primary antibody is added, followed by the secondary donkey anti-mouse Qdot 525 label. Afterward, all remaining markers (CD34, CD45, CD73, CD90) are added, as the remaining markers are pre-conjugated to fluorophores.
19. Samples, receiving STRO-1 and CD105, require secondary labeling with Alexa Fluor 568 and Qdot 525, respectively.
20. Positive identification of cell markers are defined by a fluorescent emission that exceeds the fluorescent threshold of cells stained with corresponding isotype (negative) controls.
21. It is highly recommended that no less than 20,000 events be analyzed for each sample. In addition, it is recommended that samples be analyzed at least 3×.
22. Pure RNA has a 260/280 absorbance ratio ~2.0 (1.9–2.1). Any sample that has a 260/280 ratio under 1.9 has contaminants and may not provide reliable data in downstream applications [23].
23. The 260/230 ratio is a secondary measurement of nucleic acid purity. Pure nucleic acids should have a 260/230 ratio between 2.0 and 2.2.

    - If the 260/280 absorbance ratio is below 1.8, check the 260/230 ratio. If the 260/230 ratio is below 2.0, then there may be protein contaminates (*see* [24]).

- If both the 260/280 ratio and the 260/230 ratio are below ~2.0 and 2.0, respectively, then the pH of the blanking solution may have shifted. If the pH of the blanking is slightly below 7.5, it can shift the 260/280 and 260/230 ratios. To determine if pH is affecting the 260/280 ratio, resuspend 1 μL of the RNA sample in 2 μL of TE Buffer (Tris and EDTA). Run TE Buffer as the "Blank" on the NanoDrop 2000, then test the RNA sample suspended in TE Buffer (*see* [24])
- If the 260/280 and 260/230 ratio are still outside of recommended limits, then there are most likely contaminants in RNA sample. The RNA sample can be re-cleaned with the components of a Qiagen RNeasy Kit (*see* Subheading 2.6, **step 1**). To do this, bring the total volume of RNAse-Free water that the RNA sample is suspended in up to 100 μL. If using a Qiagen RNeasy Kit, add 350 μL of RLT buffer and 200 μL of 100 % EtOH. Mix well, transfer to a brand new RNeasy column, and repeat according to Subheading 2.6, **step 1**. Re-test the RNA sample on the NanoDrop 2000 (*see* [24]).

24. *Pure cDNA has a 260/280 absorbance ratio between 1.8 and 2.0.* Any sample that has a 260/280 ratio under 1.8 or over 2.0 has contaminants and may not provide reliable data in downstream applications.
25. Tissues are preserved once embedded in paraffin and bonded to a slide. When ready to proceed with immunohistochemistry, deparaffinize tissues and keep moist. *Do not* allow tissues to dry out.
26. Reserve a set of samples to incubate only with secondary antibody label to reveal non-specific binding.

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References

1. Chen J, Steit A (2013) Introduction of the inner ear: stepwise specification of otic fate from multipotent progenitors. Hear Res 297: 3–12
2. Collado MS, Holt JR (2009) Can neurosphere production help restore inner ear transduction? Proc Natl Acad Sci U S A 106:8
3. Dhara SK, Gerwe BA, Majumder A, Dodla MC, Boyd NL, Machacek DW, Hasneen K, Stice SL (2009) Genetic manipulation of neural progenitors derived from human embryonic stem cells. Tissue Eng Part A 15:3621–3634
4. Dhara SK, Majumder A, Dodla MC, Stice SL (2011) Nonviral gene delivery in neural progenitors derived from human pluripotent stem cells. Methods Mol Biol 767:343–354
5. Wang L, Ott L, Seshareddy K, Weiss ML, Detamore MS (2011) Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells. Regen Med 6:95–109

6. Wataya T, Muguruma K, Sasai Y (2008) Human pluripotent stem cell and neural differentiation. Brain Nerve 60:1165–1172
7. Koehler KR, Hashino E (2014) 3D mouse embryonic stem cell culture for generating inner ear organoids. Nat Protoc 9:1229–1244
8. Koehler KR, Mikosz AM, Molosh AI, Patel D, Hashino E (2013) Generation of inner ear sensory epithelia from pluripotent stem cells in 3D culture. Nature 500:217–221
9. Longworth-Mills E, Koehler KR, Hashino E (2015) Generating inner ear organoids from mouse embryonic stem cells. Methods Mol Biol 1341:391–406
10. Ohnishi H, Skerleva D, Kitajiri S, Sakamoto T, Yamamoto N, Ito J, Nakagawa T (2015) Limited hair cell induction from human induced pluripotent stem cells using a simple stepwise method. Neurosci Lett 599:49–54
11. Yamahara K, Yamamoto N, Nakagawa T, Ito J (2015) Insulin-like growth factor 1: a novel treatment for the protection or regeneration of cochlear hair cells. Hear Res 330:2–9
12. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG (2002) Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A 99:12600–12605
13. Barry F, Boynton RE, Liu B, Murphy JM (2001) Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 268:189–200
14. Romanov YA, Darevskaya AN, Merzlikina NV, Buravkova LB (2005) Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization, and differentiation potentialities. Bull Exp Biol Med 140:138–143
15. Rose RA, Jiang H, Wang X et al (2008) Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. Stem Cells 26:2884–2892
16. Bailey M, Wang L, Bode C, Mitchell K, Detamore MS (2007) Comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. Tissue Eng 13:2003–2010
17. Detamore MS (2013) Human umbilical cord mesenchymal stromal cells in regenerative medicine. Stem Cell Res Ther 4:142
18. Devarajan K, Forrest ML, Detamore MS, Staecker H (2013) Adenovector-mediated gene delivery to human umbilical cord mesenchymal stromal cells induces inner ear cell phenotype. Cell Reprogram 15:43–54
19. Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D, Medicetty S (2003) Matrix cells from Wharton's jelly form neurons and glia. Stem Cells 21:50–60
20. Baksh D, Yao R, Tuan RS (2007) Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 25:1384–1392
21. Mellott AJ, Godsey ME, Shinogle HE, Moore DS, Forrest ML, Detamore MS (2014) Improving viability and transfection efficiency with human umbilical cord Wharton's jelly cells through use of a ROCK inhibitor. Cell Reprogram 2:91–97
22. Mellott AJ, Devarajan K, Shinogle HE et al (2015) Nonviral reprogramming of human Wharton's jelly cells reveals differences between ATOH1 homologues. Tissue Eng Part A 21:1795–1809
23. Bustin SA, Mueller R (2005) Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci (Lond) 109:365–379
24. Qiagen (2010) Critical factors for successful real-time PCR [E-Reader Version]. Accessed from https://www.qiagen.com/us/resources/resourcedetail?id=f7efb4f4-fbcf-4b25-9315-c4702414e8d6&lang=en