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In Vitro Time-lapse Live Cell Imaging to Explore Cell Migration Toward the Organ of Corti

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TITLE:

In Vitro Time-lapse Live-Cell Imaging to Explore Cell Migration Toward the Organ of Corti

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KEYWORDS:

Organ of Corti, time-lapse confocal microscope, mesenchymal stem cell, migration, hair cells, supporting cells, immunolabeling, coculture system

SUMMARY:

In this study, we present a real-time imaging method using confocal microscopy to observe cells moving toward damaged tissue by ex vivo incubation with the cochlear epithelium containing the organ of Corti.

ABSTRACT:

To study the effects of mesenchymal stem cells (MSCs) on cell regeneration and treatment, this method tracks MSC migration and morphological changes after co-culture with cochlear

epithelium. The organ of Corti was immobilized on a plastic coverslip by pressing a portion of the Reissner's membrane generated during the dissection. MSCs confined by a glass cylinder migrated toward cochlear epithelium when the cylinder was removed. Their predominant localization was observed in the modiolus of the organ of Corti, aligned in a direction similarly to that of the nerve fibers. However, some MSCs were localized in the limbus area and showed a horizontally elongated shape. In addition, migration into the hair cell area was increased, and the morphology of the MSCs changed to various forms after kanamycin treatment. In conclusion, the results of this study indicate that the coculture of MSCs with cochlear epithelium will be useful for the development of therapeutics via cell transplantation and for studies of cell regeneration that can examine various conditions and factors.

INTRODUCTION:

Hearing loss can occur congenitally or can be caused progressively by several factors, including aging, drugs, and noise. Hearing loss is often difficult to treat because it is very challenging to restore impaired function once the hair cells responsible for hearing are damaged[1]. According to the World Health Organization, 461 million people worldwide are estimated to have hearing loss, which accounts for 6.1% of the world's population. Of those with hearing loss, 93% are adults, and 7% are children.

A number of approaches have been attempted to treat hearing loss; notably, a regeneration approach using MSCs has emerged as a promising treatment. When tissue is damaged, MSCs are naturally released into the circulatory system and migrate to the injury site where they secrete various molecules to form a microenvironment that promotes regeneration[2]. Hence, it is important to develop a method to treat damaged tissues through the migration of externally implanted MSCs to target organs and their subsequent secretion of molecules that cause potent immune regulation, angiogenesis, and anti-apoptosis to enhance the restoration of damaged cell function[3,4,5].

The homing process in which MSCs migrate to damaged tissues may be the most important obstacle to overcome. MSCs have a systemic homing mechanism with sequential steps of tethering/rolling, activation, arrest, transmigration/diapedesis, and migration[6,7,8]. Currently, efforts are underway to identify ways to improve these steps. Various strategies, including genetic modification, cell surface engineering, in vitro priming, and magnetic guidance, have been tested[6,7]. In addition, several attempts have been made to promote the protection and regeneration of auditory hair cells by homing MSCs to the site of damaged cochlea. However, tracking MSCs in vivo is time-consuming and labor-intensive and requires highly specialized skills[9].

To solve this problem, a method was developed to observe the homing of MSCs in the cochlea through time-lapse confocal microscopy that photographs the migration of cells over several hours (**Figure 1**). It was developed in the early 20th century and has recently become a powerful tool for studying migration of specific cells.

[place Figure 1 here]

PROTOCOL:

All research protocols involving ICR mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University at Wonju College of Medicine. Experiments were performed according to the Code of Ethics of the World Medical Association. In this protocol, pregnant ICR mice were kept in a 12/12 h light/dark cycle with free access to food and water.

1. Cochleae dissection

1.1. Sterilize the laminar flow tissue culture hood by turning on the ultraviolet light for ~30 min, and spray all surfaces with 70% ethanol prior to use. Allow the surfaces to dry.

1.2. Place dissection instruments in 70% ethanol for 10 min, and dry before using.

1.3. Use an operating blade to decapitate postnatal 3–4 day-old mice (**Figure 2A**).

1.4. Place the skull under a stereomicroscope in the laminar flow hood, and soak the tissue in 70% ethanol.

1.5. Quickly soak the tissue in tissue dissection solution (1x Hank's Balanced Salt Solution, 1 mM HEPES) to remove the ethanol.

1.6. Cut the centerline of the skull with a surgical blade (**Figure 2B,C**).

1.7. Expose the skull by pulling down the skin anteriorly and cutting the external auditory canal of the ear (**Figure 2D**).

1.8. Cut from the anterior to the posterior part of the cranium across the eye line (**Figure 2E**).

1.9. Open the skull and remove the forebrain, cerebellum, and brainstem with blunt forceps (**Figure 2F,G**).

1.10. Using micro forceps, separate the cochlea from the temporal bone (**Figure 2H**).

1.11. Transfer the cochleae to a Petri dish containing tissue dissection solution.

1.12. Carefully dissect all of the cochlear otic capsule, leaving only the internal cochlear soft tissue (**Figure 2I,J**).

1.13. Hold the modiolus of the cochleae with forceps and the cochlear duct with another pair of forceps, and slowly separate the two tissues (**Figure 2K**).

1.14. Remove the stria vascularis and tectorial membrane by gently peeling them away (**Figure 2L,M**).

1.15. Place a sterilized plastic coverslip in new tissue dissection solution, and then place the organ of Corti on a coverslip of 9 mm diameter, making sure that the basilar membrane faces downward (**Figure 2N–P**).

1.16. Immobilize the tissue by pressing the Reissner's membrane and the remaining modiolus tissue onto the coverslip with forceps (**Figure 2N–P**).

1.17. Transfer the coverslip with the embedded tissue to the center of a confocal dish of 35 mm diameter.

1.18. Place the glass cloning cylinder on the dish, with the cochlear explant positioned in the center of the dish, and add 100 µL of explant culture medium (DMEM/F12, 10% fetal bovine serum (FBS), 1% N2 supplement, ampicillin (10 µg/mL))[10] inside the cylinder (**Figure 2Q**).

1.19. Plate 5×10^3 cells of mouse bone marrow-derived green fluorescent protein (GFP)-tagged MSCs in 2 mL of culture medium (45% DMEM + 45% DMEM/F12, 10% FBS, 1% N2 supplement, 10 µg/mL of ampicillin) outside the glass cylinder (**Figure 2R**).

1.19.1. When the MSCs are 80–90% confluent, passage them by detaching them with trypsin-ethylenediamine tetraacetic acid.

1.20. Carefully transfer the confocal dish to a humidified incubator and incubate overnight at 37 °C in a 5% CO_2 atmosphere.

1.21. Aspirate all medium inside and outside the cylinder, and then remove the glass cylinder from the confocal dish.

1.22. Add 2 mL of fresh culture medium to the confocal dish, and incubate the tissue culture dish in a humidified incubator until ready for analysis.

[Place Figure 2 here]

2. Time-lapse imaging

2.1. For the experiments presented here, use a confocal microscopy system with a stage top incubator system.

2.2. Turn on the confocal microscope, fluorescent light, and computer.

2.3. Set the conditions of the stage-top incubator placed on the stage of the confocal microscope to 37 °C and 5% CO_2 atmosphere.

2.4. Place the sample dish on the dish fixing vessel, cover with the dish fixing lid, and close the chamber with the top heater lid.

2.5. Adjust the zoom and focus to localize the organ of Corti and MSCs in the field of view.

2.6. Open the image processing software. Under the **locate** option, select a **20x Plan-Apochromat objective (numerical aperture 0.8)** and a **0.5x crop area**.

2.7. Under **Acquisition**, click on **smart setup** and select **EGFP**.

2.8. Open the **channel** tab under **Acquisition**, and set the **laser power** to **0.2%**, the **pinhole** to **44 µm**, the **master gain** to **750 V**, and the **digital gain** to **1.0**.

2.9. Click on **ESID** under **imaging setup**, and set the **ESID gain** to **4** and the **digital gain** to **7.5**.

2.10. Click on **Tiles** and **stake** to produce 210 tiles.

2.11. Open **Focus strategy** and select the **focus mode**.

2.12. Under **time series**, set the **duration** to **24 h** and the **interval** to **10 min**.

2.13. Under **Acquisition**, set the **frame size** to **512 x 512 pixels**, **scan speed** to **8**, the **direction** to **bidirectional**, the **averaging** to **4x**, and the **bits per pixel** to **16**.

2.14. Click on **start experiment** to begin the experiment.

3. Image file modification

3.1. Under **processing**, click on **stitching**, and set the **minimal overlay** to **5%** and the **maximal shift** to **10%**.

3.2. Click on **movie export**, set **uncompressed**, and set the **speed** to **7.5**.

4. Immunostaining

4.1. Aspirate the medium carefully, and wash the sample twice with phosphate-buffered saline (PBS) for 5 min.

4.2. Fix the sample with 4% formalin in PBS for 15 min, and wash the sample 3 times with PBS for 5 min.

4.3. Permeabilize the sample in 0.1% Triton X-100 in PBS for 10 min, and wash 3 times with PBS for 5 min.

4.4. Add 250 µL of phalloidin-iFluor 647 reagent (1:1000 dilution in PBS), and incubate the sample for 1 h at room temperature on the shaker.

4.5. Wash the sample 3 times with PBS for 5 min.

4.6. Transfer the coverslip onto the glass slide, and add 2 drops of mounting solution.

4.7. Place a coverslip on the slide gently.

4.8. Seal the coverslip with clear nail polish and store at 4 °C in the dark until cells are observed.

4.9. Image the slide using a confocal microscope with an appropriate filter at excitation/emission (Ex/Em)=650/665 nm for phalloidin and at Ex/Em=488/507 nm for EGFP.

REPRESENTATIVE RESULTS:

In vitro migration of MSCs in three-dimensional mode has been assessed by a Transwell system or by the traditional wound healing method to observe migration in two-dimensional (2D) mode[11]. The organ of Corti is a complex structure composed of various cells such as Boettcher cells, Claudius cells, Deiters cells, pillar cells, Hensen's cells, outer hair cells, inner hair cells, nerve fibers, basilar membrane, and reticular lamina[12]. When MSCs are transplanted into tissue composed of such complex cells, the use of technology to ascertain where cells are recruited and stabilized is pivotal to understand the mechanism of cell therapy and regeneration using MSCs. In addition, to determine how MSCs are localized in the presence or absence of damage, a 2D method such as a wound healing method is more suitable than a Transwell system in which cells move randomly downward in one direction.

In this study, the two-dimensional path of MSCs was tracked in a wound healing method by simply using a glass cylinder as a barrier between the MSCs and the organ of Corti and removing the cylinder after the MSCs are attached. When the organ of Corti was cultured, fibroblasts grew very quickly outward from the outer hair cells present in the outermost layer (**Figure 3**, **Video 1**). Therefore, most GFP-labeled MSCs seemed to be pushed out by fast-growing fibroblasts (**Video 1**). Nonetheless, some GFP-labeled MSCs penetrated the layer of fibroblasts and successfully migrated to the organ of Corti (**Figure 3**, **Video 1**).

[Place Figure 3 & Video 1]

The results from 72 h of incubation showed that MSCs migrated to the organ of Corti and were mainly localized in the modiolus and the area where the cochlear nerve fibers separated from

the modiolus were gathered; the morphology of the MSCs was altered to a radial shape similar to that of nerve fibers (**Figure 4B**). Intriguingly, cells were transformed into a linear shape along the limbus line rather than the hair cell area (**Figure 4E**, see arrow). Although the apical and basal ends of the organ of Corti were combined to prevent the MSCs from moving to the medial side of basilar membrane, MSCs were able to migrate and grow in the nerve fiber collection area by penetrating the various cell layers and physical barriers (**Figure 4E**). When damage to hair cells was induced by treatment with 1 mM kanamycin for 16 h, and cells were cultured for a further 72 h after the removal of kanamycin, MSCs were localized not only in the modiolus, but also in the outer hair cells (**Figure 4C,D**).

[Place Figure 4 here]

These results indicate that the experimental system demonstrated in this study would be a useful test tool for developing cell therapeutic strategies using MSCs and can be applied specifically to study hearing loss caused by various factors.

FIGURE AND TABLE LEGENDS:

Figure 1: Graphical abstract. (**A**) After the dissected organ of Corti is adhered on a plastic coverslip using forceps, the coverslip is placed on a 35 mm glass-bottomed confocal microscopic dish, and (**B**) the glass cylinder is positioned. (**C**) After filling the inside of the glass cylinder with medium, (**D**) GFP-labeled MSCs with medium are added carefully outside the cylinder. (**E**) After incubation overnight, (**F**) the glass cylinder is removed, and images are taken with a confocal microscope. Abbreviations: GFP = green fluorescent protein; MSCs = mesenchymal stem cells.

Figure 2. Dissection of a mouse cochlea and coculture of the organ of Corti and MSCs. (**A**) Decapitation of mouse, (**B**) and (**C**) midline sagittal dissection of the head, (**D**) and (**E**) coronal dissection of the brain, (**F**) and (**G**) removal of the brain and temporal bone, (**H**) the cochlea, (**I**) removal of the bony cochlear wall, (**J**) isolation of the cochlea, (**K**) separation of the cochlear duct from the modiolus, (**L**) separation of stria vascularis (SV) and spiral ligament (SL) from the organ of Corti, (**M**) removal of the tectorial membrane, (**N–P**) fixation of the cochlea on a plastic cover slip, (**Q**) location of coverslip and glass cylinder in the confocal dish, (**R**) inoculation of MSCs. White scale bar (**A–E**) = 1 cm; orange (**F, G, P**) and yellow scale bar (**H, I**) = 1 mm; green scale bar (**J–O**) = 0.5 mm.

Figure 3. Confocal image of the organ of Corti and GFP-labeled MSCs. After the removal of the glass cylinder and an additional 4 h of incubation, fixation and staining were performed. Scale bar =100 µm. Abbreviations: GFP = green fluorescent protein; MSCs: mesenchymal stem cells.

Figure 4. Confocal images of the organ of Corti and GFP-labeled MSCs. (**A**) After the removal of the glass cylinder and an additional 16 h of incubation, an image was taken after fixation and staining. Scale bar =200 µm. (**B**) MSCs were predominantly distributed in the modiolus area, scale bar =20 µm. After the removal of the glass cylinder from the dish, the cells were further incubated with 1.0 mM kanamycin for 16 h, cultured with kanamycin-free medium for 72 h, and then fixed

and stained. Images of the (**C**) outer hair cell region, scale bar= 20 µm; (**D**) modiolus region, scale bar= 50 µm; and (**E**) whole cochlea, scale bar= 100 µm.

Video 1: Time-lapse confocal microscopy of the organ of Corti and GFP-labeled MSCs. After the removal of the glass cylinder and an additional 4 h of incubation, fixation and staining were performed. Scale bar =100 µm. Abbreviations: GFP = green fluorescent protein; MSCs: mesenchymal stem cells.

DISCUSSION:

Transplantation of MSCs into damaged sites to promote the regeneration of damaged cells has been extensively studied, and the therapeutic effect is evident. The transplantation and subsequent differentiation of MSCs have been reported to restore hearing in rats with hearing loss induced by 3-nitropropionic acid[13]. Although Lee et al. applied MSCs to human beings trans-venously, they did not achieve any significant improvement in hearing[14]. Until recently, nearly 12 experiments were conducted to restore hearing in the rodent model by MSC transplantation. Although the result is somewhat unclear because of heterogeneity, there is some indication that MSCs can promote hearing.

Various growth factors and chemokines related to chemoattraction are involved in the migration of transplanted stem cells to damaged areas, regardless of heterogeneity or homogeneity[15]. Chemokines are key regulators of cell migration for tissue homeostasis, immune responses, and wound healing. Chemokine-induced chemoattraction in vivo might not work in the same way as it does in vitro due to the differences between in vivo and in vitro experimental conditions. Although in vitro experimental conditions have their own limitations, it is very difficult to conduct in vivo studies to promote efficient migration, recruitment, and regeneration of cells[16].

Although the tracking of MSCs transplanted for hearing restoration is important in regenerative medicine research, in vivo experiments, especially those involving hearing, are labor-intensive in most cases and are therefore extremely difficult to perform with large sample sizes. Hence, the homogeneity of the results is low, and the bias is severe. Therefore, it would be efficient to first study such migration-based events under in vitro conditions. Most in vitro studies on cell migration have been performed by using Transwell or wound healing methods.

A coincubation method of explanting cochleae with MSCs was established to track the migrating stem cells, which recognize injury sites and move toward them. Most studies have used a method of attaching an organ of Corti on glass coverslips coated with β-mercaptoethanol after explantation. Although the same method was used here, it was difficult to examine the tissues because they often detached or rolled up from the coverslip. Therefore, a new trial was performed to make it easier to monitor the tissue. First, an organ of Corti was placed on a plastic coverslip, and then the tissue was immobilized on the coverslip by pressing the remaining portion of the Reissner's membrane generated during the dissection using forceps.

This enabled the stable attachment of the organ of Corti to the coverslip so that an image could be acquired successfully by confocal microscopy. Although the tissue culture method presented here is an inexpensive and time-saving, development of proficiency in dissection does take some time. Known tissue culture methods include the attachment of the tissue by coating the coverslip with poly-L-ornithine and laminin[16]; applying organotypic cell culture inserts that do not require adhesive or coating materials[17] thereby eliminating the need for adhesive substances, which reduces explant damage; and using a tissue adhesive composed of proteins extracted from marine mussels[10]. This protocol requires neither coating materials nor organotypic membranes nor tissue adhesives; an inexpensive plastic coverslip is sufficient. Better images can be acquired if an area of 8 mm x 8 mm is considered along with a Z-stack of the tissue thickness of the organ of Corti. Considering the vast amount of data to be stored and the appropriate time for time lapse, images of whole cell movement were acquired, focusing on the MSCs instead of the organ of Corti.

The results shown in **Figure 4** seem to suggest that both the organ of Corti and MSCs can be visualized as clear video images when the images taken are 2 mm long, and the Z-stack is combined with a time lapse. Here, although a co-culture experiment was performed with MSCs and an explant, this setting could be applied to different cell types or conditions for future studies. For example, it will help to apply this protocol to examine the effects of drugs or exosomes that protect the damage of cochlea. Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) can functionally and morphologically regenerate into mechanosensitive hair cell-like cells[18]. Therefore, this protocol can be applied to study the differentiation of iPSCs or ESCs into auditory hair cells or support cells. This ex vivo method can aid in regenerative medicine to restore hearing.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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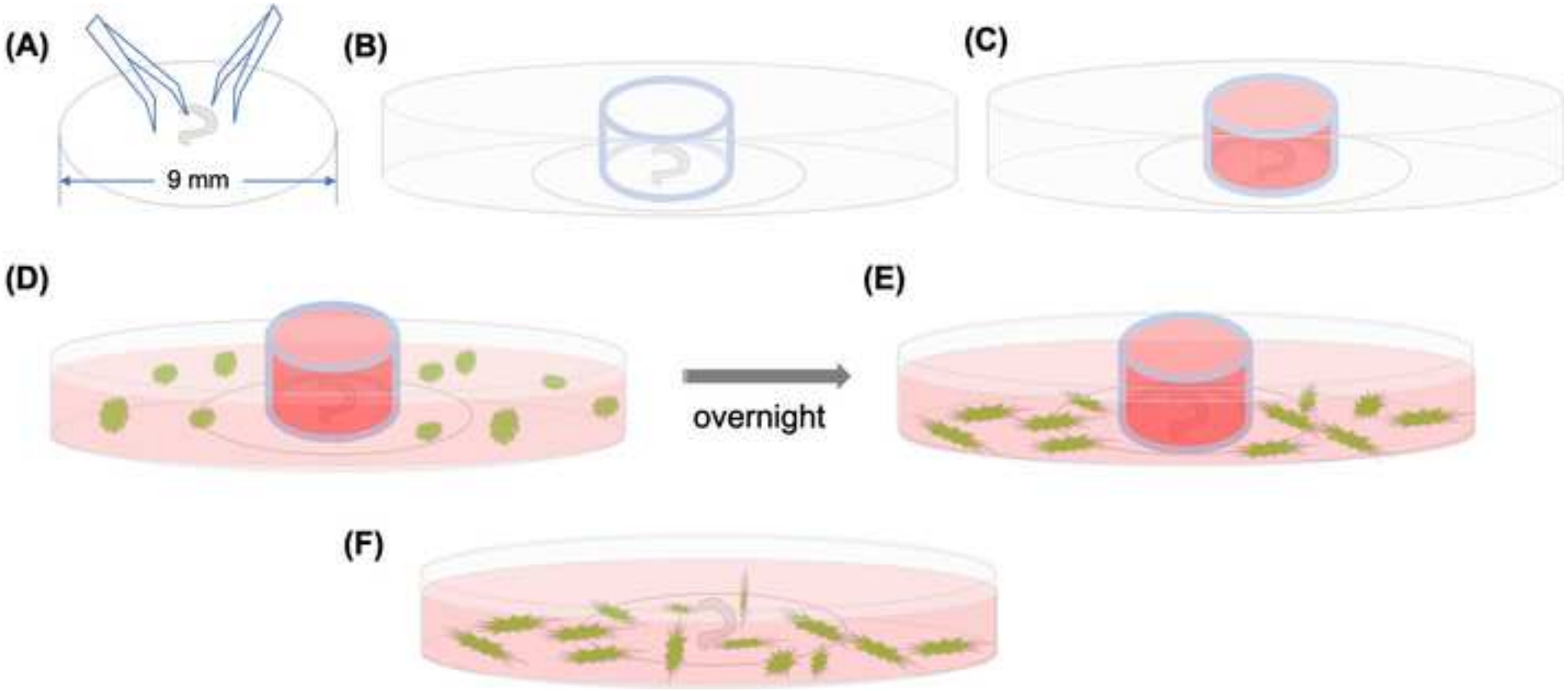

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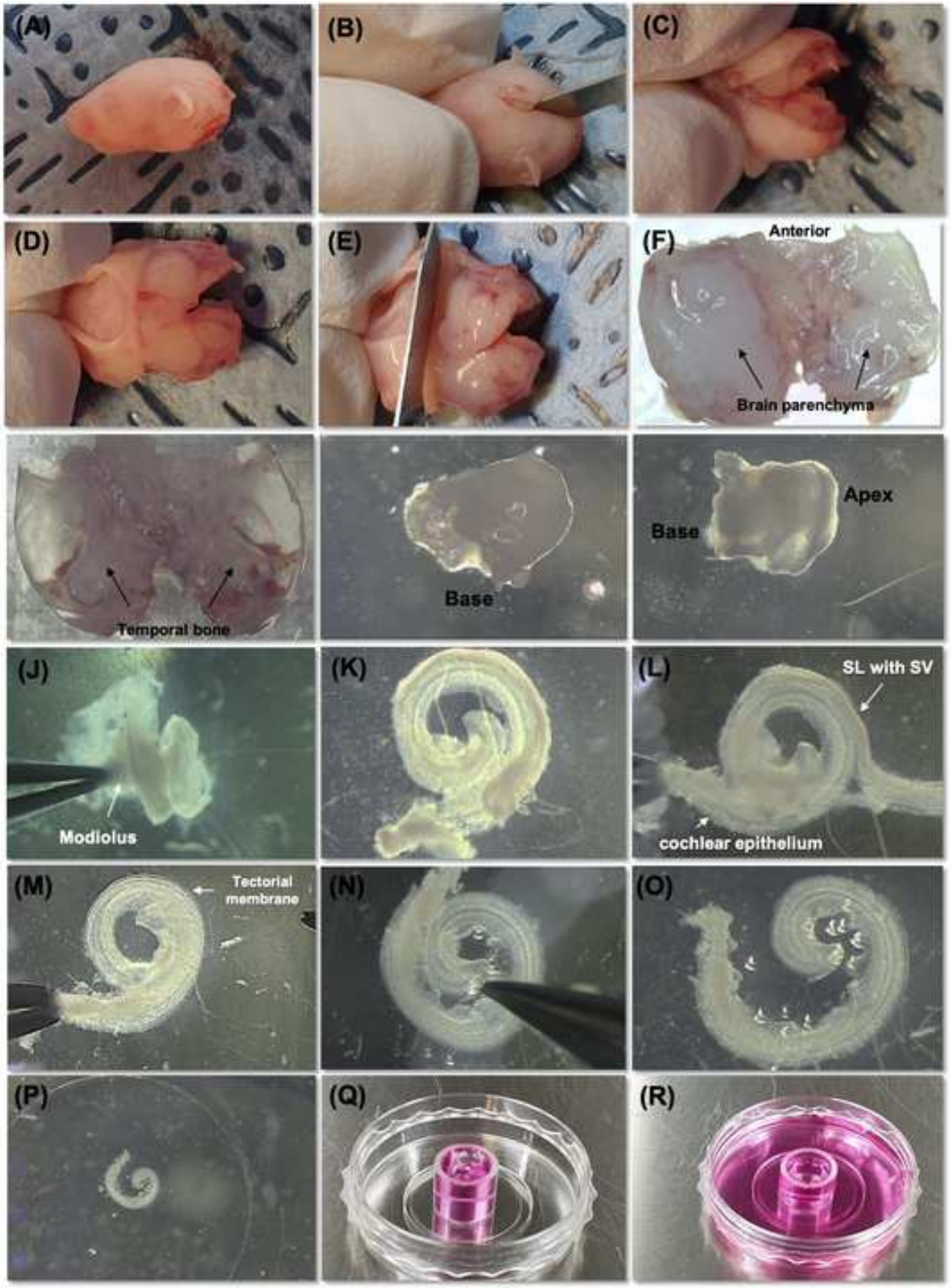

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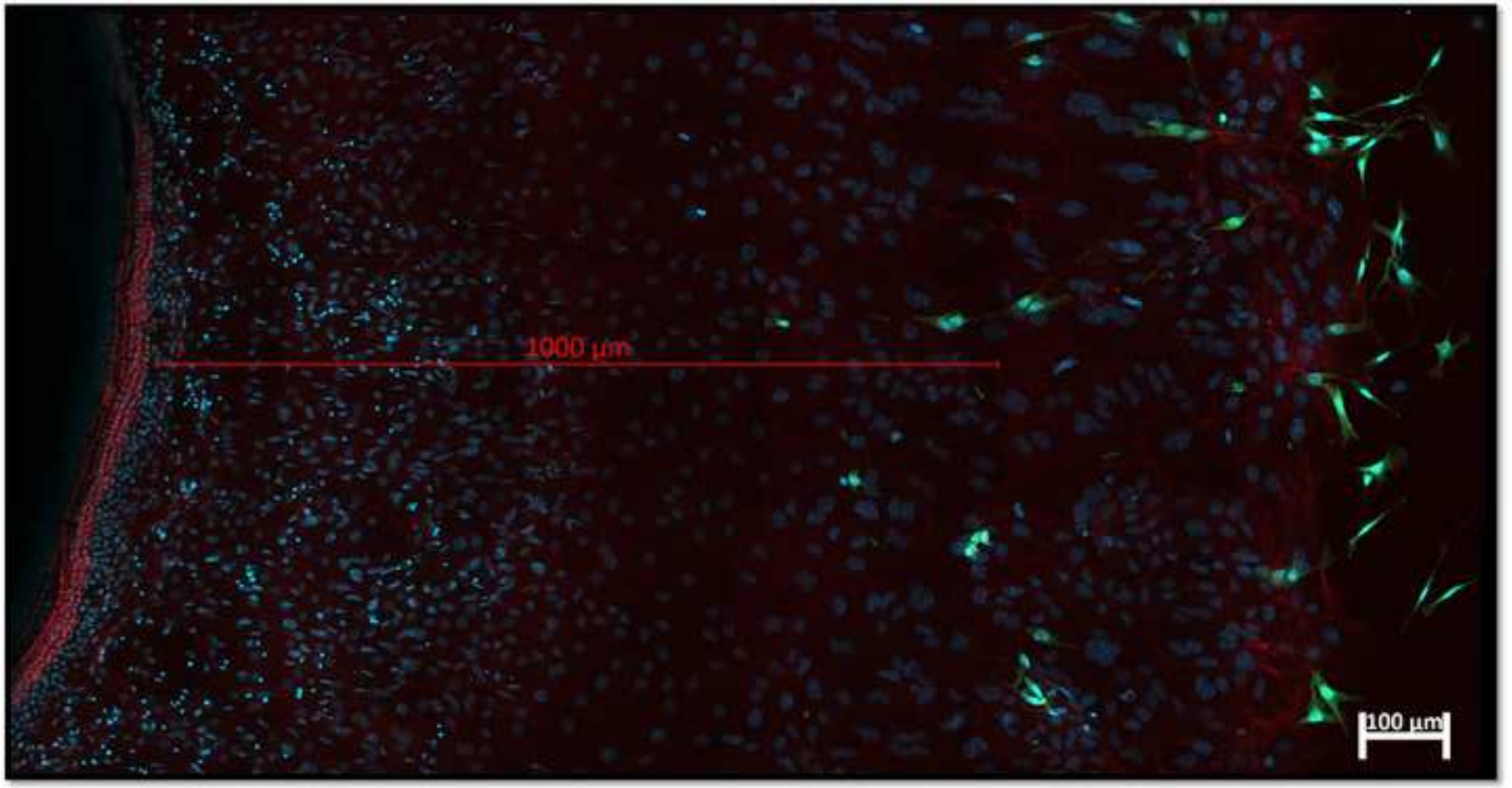

Figure 4

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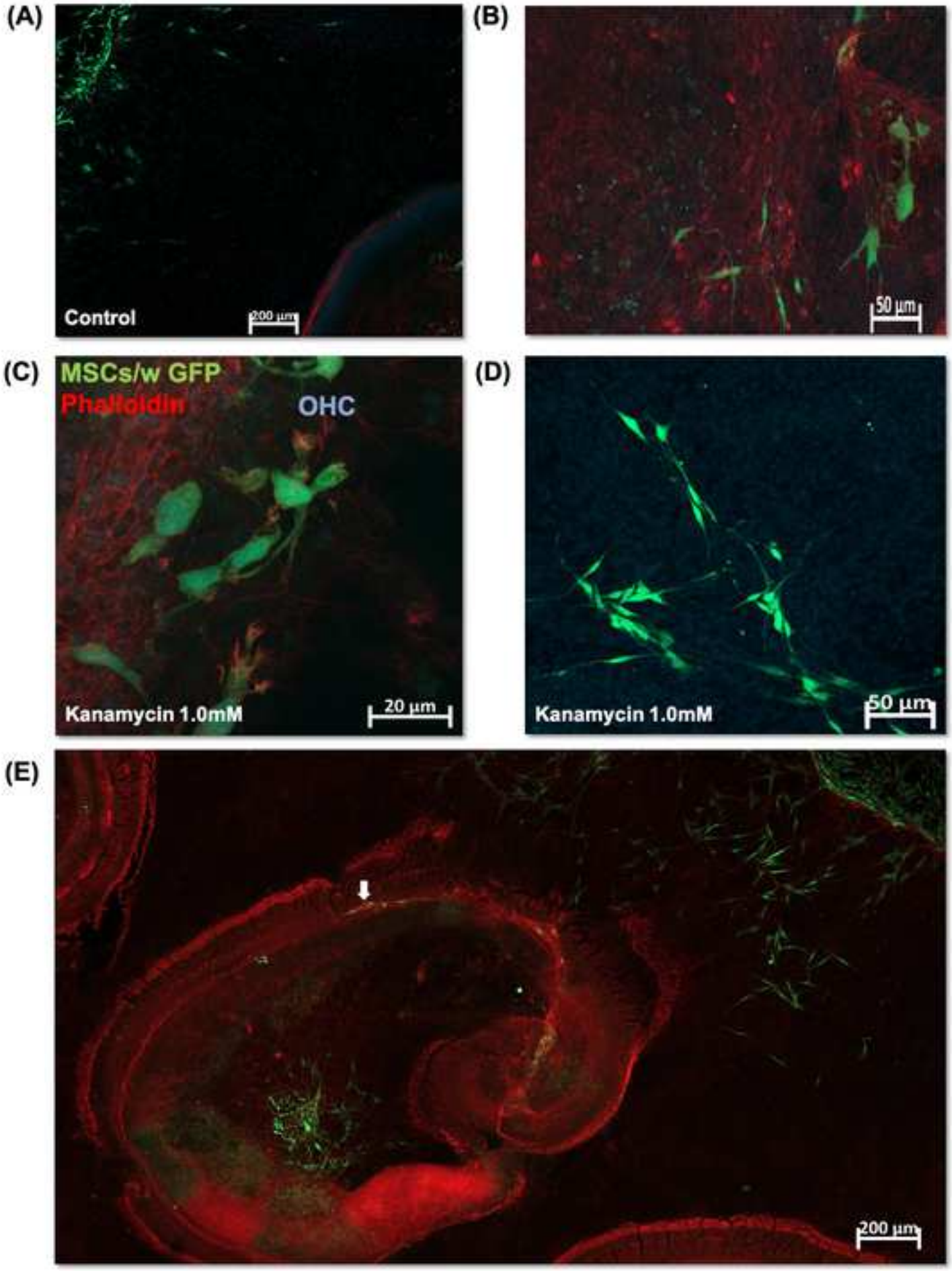

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Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10X PBS Buffer	GenDEPOT	P2100-104	
4% Formalin	T&I	BPP-9004	
Ampicillin	sigma	A5354-10ml	
BSA	sigma	A4503-100G	
confocal dish	SPL	200350	
confocal microscope	ZEISS	LSM800	
coverslip	SPL	20009	
DMEM/F12	Gibco	10565-018	
Fetal Bovine Serum	Thermo Fisher scientific	16140071	
Fluorsheild with DAPI	sigma	F6057	
Forcep	Dumont	0508-L5-P0	
HBSS	Thermo Fisher scientific	14065056	
HEPES	Thermo Fisher scientific	15630080	
N2 supplement	Gibco	17502-048	
Phalloidin-iFluor 647 Reagent	abcam	ab176759	
Stage Top Incubator	TOKAI HIT	WELSX	
Strain C57BL/6 mouse messenchymal stem cells with GFP	cyagen	MUBMX-01101	
Triton X-100	sigma	T8787	

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Title: *In Vitro* Time-lapse Live Cell Imaging to Explore Cell Migration toward the Organ of Corti

It is with excitement that I resubmit to you a revised version of manuscript, *In Vitro* Time-lapse Live Cell Imaging to Explore Cell Migration toward the Organ of Corti for the *JoVE*. Thank you for giving me the opportunity to revise and resubmit this manuscript. In keeping with my last communication with you, I am resubmitting this revision before the agreed upon deadline, Oct 29, 2020. I appreciate the time and detail provided by each reviewer and by you and have incorporated the suggested changes into the manuscript and figure to the best of my ability. The manuscript has certainly benefited from these insightful revision suggestions. I look forward to working with you and the reviewers to move this manuscript closer to publication in the *JoVE*. I have responded specifically to each suggestion, beginning with your own. To make the changes easier to identify where necessary, I have numbered them.

I am looking forward to your reply.

Thank you for your consideration of this manuscript.

Sincerely,

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The revisions made after carefully considering the comments of the reviewers and editor are as follows. (Note: reviewer comments are in italics; our responses are in light blue.)

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: As you recommended, each paragraph of the introduction section has been modified to consist of three or more sentences.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions,

numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

: As commented, the protocol has been revised to make it easier to take videos, and detailed explanations have been added so that the described experimental method can be easily followed, especially in parts of cochlear dissection and time-lapse imaging.

5. Please specify the euthanasia method without highlighting it.

: We appreciate your kind comments and have added information on ethical handling of animals (protocol 1.1-1.3) and sterilization of dissection instruments (protocol 1.4-1.6).

6. 1.14: By fixing, do you mean simply immobilizing the tissue on the coverslip or fixing with formaldehyde? If not the latter, please use the word "immobilize" instead of "fix'.

: Since 1.14 refers to the process of physically immobilizing the tissue, it is more appropriate to substitute immobilize instead of fix. Thanks for your thoughtful comments.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: LSM-800 confocal Zeiss microscope, Tokai Hit incubator system, Zen Software

: The commercial language included in 2.1 and 2.6 of the protocol sections has been replaced by a generic term as you mentioned. Thank you for making the right point appropriate for JoVE's purpose.

8. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

: We highlighted essential step for making video in protocol text.

9. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

: The critical step in this protocol is to attach the organ of Corti well to the coverslip using sterile plastic coverslip. It is important to handle the tissue carefully at this step in order to obtain a clean and orderly image of the organ of Corti. (Line 281-289)

b) Any modifications and troubleshooting of the technique

: In this protocol, only the method using MSCs and organ of Corti is introduced. If iPSCs or ESCs instead of MSCs will be applied to this method, it will be very useful for differentiation studies of stem cells transplanted into tissues. The related contents are mentioned in line 300 through 307 of the discussion section.

c) Any limitations of the technique

MSCs are very thin cells that grow attached to the bottom. On the other hand, the organ of Corti is a thick tissue made up of multilayered cells. When taking a confocal microscope, it is not easy to get sharp images for both. In order to obtain a clear image, it is good to minimize the area to be photographed and use the Z-stack to focus on the organ of Corti. However, if you focus on migration, the shooting area will be wide, and in this case, it is better to shoot focusing the MSC. The related contents are mentioned in line 295 through 300 of the

discussion section.

d) The significance with respect to existing methods

: Existing in vitro methods to study migration include trans-well or wound healing methods. It is meaningful that our method allows us to identify the part that MSCs likes to locate. The related contents are mentioned in line 183 through 201. In terms of culture of explant, the significance of our method compared to the existing method is that it achieves a reduction in time and cost and simplification of steps. The related contents are mentioned in line 286 through 294 of the discussion section.

e) Any future applications of the technique

: We intend to study the effect of MSCs on the regeneration of hair cells by applying this method to ototoxic damaged mouse model. And it would be good if this technique was applied to study substances or conditions that affect the homing effect of stem cells. The related contents are mentioned in line 300 through 307 of the discussion section.

10. There seem to be three important aspects of your manuscript: the time-lapse microscopy method, the method of isolation of the organ of Corti, and the application of this MSC migration toward the organ of Corti for tissue engineering. Please introduce this last aspect in the introduction and discussion as it has only been mentioned in the abstract.

: Thanks for the helpful comment. We added more detail description about the time-lapse confocal microscopy (line 74 to 78), the isolation of organ of Corti (line 278 to 294) and the application of MSCs migration toward organ of Corti for tissue engineering (line 256 to 262, line 300 to 307).

11. Please include scale bars in the figures and legends (e.g., Figures 2,3,5).

: In the figures and legends you mentioned, we have added a scale bar to make it easier to understand. Thanks for the meaningful comment. (As Figure 2 was divided into two-Figure 2 and 3-and uploaded incorrectly, there was confusion about number. Therefore, the existing Figures 2 and 3 are combined and made into one. There is no change in the order of Legends.)

12. Please sort the Materials Table alphabetically by the name of the material.

: Each item in the materials table is arranged alphabetically based on the material name.

Reviewer #1:

Manuscript Summary:

Manuscript reads well and concise. The authors also mentioned therapeutic significance of this protocol. But they should certainly include more details specifically the biology of MSCs that they used. The movie 1 should include only the movie.

Major Concerns:

What do authors expect to see if OC explants are cultured without underlying mesenchyme, for instance, post thermolysin treatment. Are these MSCs migrating towards otic mesenchyme underlying these epithelial cells. If you see the migration post thermolysin and kanamycin treatment, then it will provide us with direct evidence of MSCs migration towards injury site.

: Thank you for your valuable comments. MSCs are multipotent cells isolated from adult bone marrow, lipid, tonsil and umbilical cord and it can be transplanted and differentiated into various tissues in vitro and in vivo. Human stem cells were transplanted into fetal sheep and differentiated into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma. When the MSCs were transplanted into postnatal animals, they were

able to differentiate into several tissue-specific cell types in response to environmental signals from different organs. In addition, until recently, approximately 12 representative studies were demonstrated that MSCs transplanted into the inner ear lead to restoration of hearing threshold in the rodent model. Although the results are somewhat unclear and heterogeneity, it has suggested that MSCs in the inner ear can go through various mechanisms and play a positive role in hearing restoration. Therefore, it shows the evidence that stem cell transplantation can be a promising treatment for hearing loss in a animal model and even human. Before starting an in vivo experiment – the result of which has a high bias and high heterogeneity – increasing the homogeneity of the result through an easily repeated experiment in ex vivo will be of great help for regenerative medicine of restoring hearing loss using MSC.

One of the important points in the field of treatment using MSC is that MSC cells move to the target cells or tissues. The main purpose of this paper is to introduce a new co-culture method of MSCs and Cochlea explant. It is also an important and meaningful investigation to analyze the degree of migration of MSC according to the degree of damage or the concentration of drugs when ototoxicity such as damage of hair cells, hearing loss is cause by kanamycin or thermolysin. However, our study is more focused on introducing new methods of measuring the migration and pathway of MSC for various drugs or damages in real time. Although many researchers have investigated to increase the mobility of these MSC and homing efficiency to the target tissue, the evolution and development of stem cell research in the field of otology is still very slow due to the aforementioned barriers. Through this, we hope that it will be a technology that helps other researchers who are trying to find a way to efficiently treat sensorineural hearing loss such as an age-related hearing loss and an ototoxicity related hearing loss through stem cells.

Minor Concerns:

There is abrupt breakage of words here and there that should be taken care of.

There is no breakage of words in our manuscript.

If there is the same problem in the revised manuscript, please let us know in detail and we will take action.

Reviewer #2

Manuscript Summary:

The manuscript provides a description for the establishment of cochlear explant cultures followed by the addition of MSCs to the cultures and subsequent imaging.

Major Concerns:

There is very limited usefulness to the protocol. Establishment of cochlear explant cultures has been published in JOVE several times and the subsequent addition of MSCs is trivial.

: Thank you for your valuable comments. Cochlea is a very small sensory organ in which interact complex electro-physiological signals. The organ of Corti is the core of cochlea, and many researchers have attempted ex vivo studies to understand the anatomy & physiology of organ of Corti. However, it is very difficult to maintain the explant cochlea in a viable state for more than 72 hours, and in general, cell loss and tissue damage often occur despite using dishes coated with expensive extracellular matrix protein. To describe in detail, first, in the process of transferring the dissected organ of Corti using a micropipette tip or in the process of removing or adding the culture medium, the organ of Corti often sticks to the inside of the tip. This leads to damage or loss of the dissected organ of Corti. Secondly, despite the use of expensive extracellular matrix solution, the attachment of the organ of Corti is still remains a difficult process. Even if organ of Corti was attached on the dish, many of them were detach during the

immunofluorescent staining. Therefore, we suggest the use of affordable coverslip in protocol section. Once the organ of Corti is attached, it does not fall of easily. We think that this is trivial but helpful tip to solve the difficulties experienced during explant culture to many researchers. At the same time, This can increase the efficiency of inner ear studies, which take a relatively longer time than other studies by simply changing the experimental method.

Tracking the migration of transplanted MSC into the inner ear and confirming the pattern of differentiation into various cells that make up the organ of Corti are the most important steps in regenerative medicine of otology. In vivo experiments, especially inner ear research involving hearing, are labor-intensive in most cases, and are extremely difficult to perform experiment with big sample size at the same time. Therefore, the homogeneity of the results is very low, and the various types of bias occur frequently. An experiment designed to overcome these limitations is an *ex vivo* experiment using an explant. In addition, the *ex vivo* experiment is an intermediate step between *in vivo* and *in vitro*, and it is possible to conduct experiments on multiple samples with a shorter time than *in vivo*. Also, since morphological changes to the target tissue can be confirmed, relatively constant results can be derived. Therefore, we expect that this study will serve as technical support for the areas that many researchers have struggled with and improve accessibility to inner ear research in the future.

Minor Concerns:

Line 40: change "organ of Corti" to "cochlear epithelium" as the explant includes more than the organ of corti.

: We modified the organ of Corti with the “cochlear epithelium” as you pointed out. Thank you for your insightful comments.

Line 104: What is the jelly bone

: The skull of the postnatal 4-day mouse is still less calcified and remains soft, so it is expressed as jelly. However, this was not appropriate from the academic point of view and was modified to be a “cochlear otic capsule”. Thank you for making our manuscript stronger with your valuable comments.

Line 108: It is very difficult to actually remove the tectorial membrane and probably not necessary.

: Thank you for your insightful comments. Removal of the tectorial membrane is not very difficult for experienced researchers. At postnatal 2 or early 3 days, tectorial membrane removal may cause hair cell loss sometimes, making it impossible to obtain adequate specimens, but P3 or 4-day mice can easily remove the tectorial membrane. The tectorial membrane is removed by itself during culture and immunostaining, but it often adheres to hair cells and it leads to interfere with the binding of antibodies or identification of hair cells under a microscope. Therefore, we recommend removing the tectorial membrane during cochlear dissection.

Line 113: suggest changing "fix" to "adhere" as fix often means to add a fixative such as paraformaldehyde.

: In the editorial comment there was also a point on word replacement, so we modified it to “immobilize”. Thank you for your insightful comments.

Line 142: The settings listed here are too specific as these will vary based on the microscope being used, culture conditions, etc.

: Since the purpose of JoVE is that viewers who have access to this manuscript and video can easily replicate the protocol, each step is described in detail. However, as you mentioned, it

may vary depending on the image processing software, microscope, and culture conditions used by researchers, but we have described the best way to time-lapse tracking MSC migrations in mouse cochlear explant models. Thank you for your grateful comments.

Line 202: there is not figure 4E and no arrow in the image.

: As Figure 2 was divided into two-Figure 2 and 3-and uploaded incorrectly, there was confusion about number. Therefore, the existing Figures 2 and 3 are combined and made into one. There is no change in the order of Legends. Also, we added an arrow to figure 4E as you pointed out. Thank you for your very thoughtful comments.

Line 203: what is meant by "to prevent the MSCs from moving inward"?

: Thank you for your valuable comments. In our experiment, the organ of Corti was placed in a round shape to better understand the direction of MSC migration. In other words, initially MSC is located lateral side of the outer hair cell and had to pass through the inner hair cell line to move inside the organ of corti. Therefore, as shown in Figure 4E, the MSC located inside the organ of Corti has moved across the hair cell. To help understand the sentence, "inward" was modified to "medial side of basilar membrane".

Line 218: as for line 113, change "fixed" to "adhered"

: We corrected the “fixed” to “adhered” as you suggested. This makes the sentence more understandable to viewers.

Figures 2f-h could use some arrows, better illustration of important landmarks, etc.

: In agreement with your opinion, we marked the anatomical position and structure as arrows in Figure 2f-h.