ウェブサイト公開

（様式 10）
（海外特別研究員事業）
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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　Bethesda, Maryland　（国名：　US　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
　発現誘導型マウスと新型超解像顕微鏡による内耳不動毛発生・修復過程の解析

3. 派遣期間：2019 年　4 月　1 日　～　2021 年　9 月　30 日（914 日間）

4. 受入機関名及び部局名
　受入機関名：　National Institutes of Health
　部局名：　Laboratory of Molecular Genetics

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**

【記載事項】
- 研究・調査実施状況及びその成果の発表・関係学会への参加状況等
- 新型コロナウイルス感染症の影響にかかる特例措置のうち、国内採用開始・採用期間延長・翌年度渡航のいずれかの適用を受けた場合は、当該措置の適用による影響等

（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

Final Report for JSPS Overseas Fellowship

Introduction

The goal of this study is to elucidate how stereocilia in the cochlea acquire structural stability and tolerate years of mechanical stimulation caused by sound waves. Stereocilia are huge F-actin bundles protruding from the apical surface of hair cells and function as mechanical switches to convert sound waves into electrical impulse of neurons. The F-actin cores in stereocilia are continuously remodeled by the polymerization and depolymerization of actin monomers and by the replenishment of other components, such as bundling proteins, barbed-end cappers, motor proteins and proteins related to mechanotransduction. However, the entire architectures stereocilia are maintained during a lifetime of a person (Jia et al., 2009).

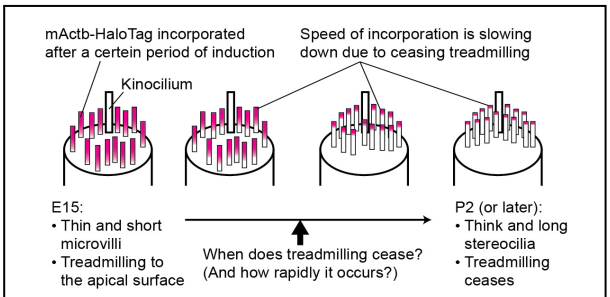

Figure 1: Overview of actin dynamics change during the transition. At a point during development, a population of microvilli cease treadmilling of F-actin and becomes mature stereocilia.

Stereocilia develop from microvilli that emerge on the nascent hair cells. Only a population of microvilli are allowed to become mature stereocilia during the development of inner ear (Figure 1). The transition process from microvilli to stereocilia, which I refer to as "microvilli-to-stereocilia transition" in this report, accompanies a change of actin dynamics. Actin filaments in microvilli treadmill toward the apical surface of hair cells while those in mature stereocilia cease treadmilling at a certain timing during the development and incorporate new actin monomers mainly at the tips (Drummond et al., 2015; Meenderink et al., 2019; Narayanan et al., 2015). I hypothesized that molecules involved in the cessation of treadmilling are crucial to cause the microvilli-to-stereocilia transition and to maintain the entire architectures of stereocilia for years despite the turnover of molecular components.

To visualize the change of actin dynamics, I initially planned to use a combination of multiplex super-resolution microscopy using fast-dissociating imaging probes, namely IRIS

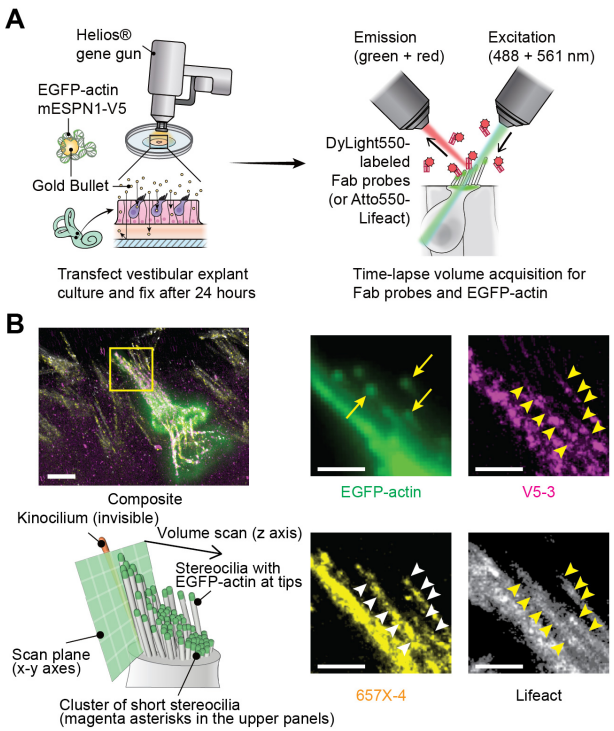

Figure 2: Three dimensional imaging of vestibular stereocilia using the IRIS technique and fluorescently-labeled Fab fragments (Fab probes). (A) Diagram illustrating the procedure. Vestibular explant cultures were transfected using a gene gun to express EGFP-actin and V5-tagged espin (mEspn1-V5). Cells expressing these two proteins were located under a dual-view light-sheet microscope, diSPIM, and imaged using Fab probes. (B) A representative multiplex three dimensional image acquired using diSPIM. The image of EGFP-actin was acquired using conventional volume scan while other images were reconstructed from 720–1,440 volume acquisitions. EGFP-actin is incorporated at the tips (EGFP-actin, arrows) while mESPN1-V5 was detected along the entire length of stereocilia (V5-3, arrowheads). Endogenous espin and F-actin were detected along the entire length of stereocilia (657X-4 and Lifeact, arrowheads). Bars, 5

(Kiuchi et al., 2015), and genetically-engineered mice with an inducible transgene to express tagged actin. While I had been supported by this fellowship, I successfully published my methodologies to identify fast-dissociating antibodies suitable for IRIS and to perform three-dimensional imaging using these antibodies (Miyoshi et al., 2012; Miyoshi et al., 2021). However, the sensitivity of IRIS was not sufficient to visualize microvilli on the apical surface of hair cells. Thus, I changed the design of transgenic mice to express actin with a self-labeling tag, HaloTag, and to visualize the microvilli-to-stereocilia transition using another cutting-edge super-resolution microscopy, STED. Here, I report my achievements during the fellowship including (1) two publications on IRIS, (2) development of mice and (3) current results on STED imaging.

Achievements

1. Three-dimensional imaging of stereocilia using IRIS technique

In the first year of fellowship, I collaborated with Dr. Hari Shroff in NIBIB/NIH to image stereocilia using the IRIS technique (Figure 2). While I was in Japan, I developed a methodology to identify fast-dissociating antibodies suitable for IRIS technique and extended the application of this technique to virtually any antigen by synthesizing fast-dissociating fluorescently-labeled Fab fragments (Fab probes) from the identified antibodies (Fab probes). However, imaging of three-dimensional structures, such as stereocilia protruding from the apical surface of hair cells, was difficult with light-sheet microscopes available in Japan. Thus, I utilized the cutting-edge light-sheet microscope developed by Dr. Hari Shroff, namely diSPIM (Wu et al., 2013), to perform three-dimensional imaging of stereocilia (Figure 2A).

I prepared vestibular explant cultures and transfected them using a gene gun to express two tagged proteins, EGFP-actin and V5-tagged espin (mEspn1-V5), to analyze how proteins are replenished to the F-actin cores of stereocilia (Figure 2B). I fixed the explant cultures after a sufficient amount of these proteins were expressed and applied anti-V5 tag Fab probe to detect mEspn1-V5. I also applied anti-mEspn1 Fab probe and Lifeact peptide probe for counterimaging of endogenous espin and F-actin. Images of V5-tag, endogenous espin, endogenous F-actin were reconstructed using Imaris image processing software and my custom-made Python script to register to conventional volume scan images of EGFP-actin. Incorporation of EGFP-actin was mainly limited the tips as previously reported, indicating that the F-actin cores are stabilized (Drummond et al., 2015; Narayanan et al., 2015). In contrast, mEspn1-V5 was detected along the entire length of stereocilia suggesting that this F-actin bundling protein can freely move the stabilized F-actin core. This finding was supported by a FRAP experiment performed in Dr. Benjamin Perrin's laboratory in Indiana University-Purdue University Indianapolis.

These results were published in two journals, Cell Reports and STAR Protocols, in 2021 (Miyoshi et al., 2012; Miyoshi et al., 2021). However, I found that imaging of nascent stereocilia using IRIS technique is still challenging even with diSPIM and other cutting-edge light-sheet microscopes. Thus, I changed the strategy after discussing with my PI, Dr. Friedman, and engineered transgenic mice using a self-labeling tag, HaloTag.

2. Development of transgenic mice to express HaloTag-fused actin

To visualize the change of actin dynamics during the microvilli-to-stereocilia transition, I established mice harboring inducible transgenes encoding mouse actin fused with HaloTag (mActb-HaloTag) (Figure 3). HaloTag is a self-labeling tag that can be labeled using a ligand conjugated with bright, robust organic fluorescent dyes, such as Janelia Fluor 549 (JF549) and 646 (JF646) (Grimm et al., 2015; Liss et al., 2015). I established a transgenic mouse line, FLEx-mActb-HaloTag, based on a CβA-FLEx backbone vector to induce mActb-HaloTag by an irreversible, two-

step Cre recombination (Figure 3A) (Franco et al., 2012). This mouse line was developed in Dr. Lijin Dong's laboratory (NEI/NIH) who is a Dr. Friedman's long-standing collaborator. Currently, I have twelve F0-founders for FLEx-mActb-HaloTag, which I have Sanger-sequencing verified and successfully bred.

Induction of mActb-HaloTag expression was tested using a Gfi1-Cre mouse line, which express Cre in hair cells at approximately E15.5 (Yang et al., 2010) (Figure 2B). I crossed FLEx-mActb-HaloTag (+) females with Gfi1-Cre (+) males and obtained pups around P3. Cochlear sensory epithalia were fixed and stained using JF549-conjugated HaloTag ligand. Mice from six F0 founder lines successfully expressed mActb-HaloTag, which include two lins that can express mActb-HaloTag in many cells, without leak expression (representative images in Figure 2B). In addition, the JF549-ligand successfully detected mActb-HaloTag incorporated in tiny microvilli (0.5–1 μm) on the apical surface of P0 inner hair cells (Figure 3C) indicating that the combination of HaloTag and the JF549-ligand is sufficiently sensitive to detect mActb-HaloTag molecules in microvilli.

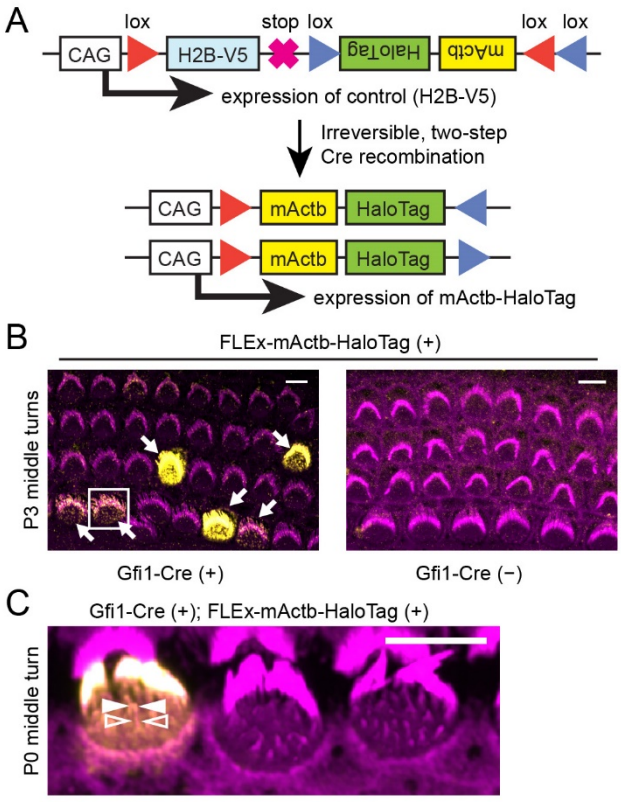

Figure 3: The FLEx-mActb-HaloTag mouse line to induce HaloTag-fused mouse β-actin (mActb-HaloTag) expression. (A) Design of transgene. Using two sets of lox sites (red and blue triangles), Cre recombination occurs irreversibly to induce mActb-HaloTag expression. (B) Cre-specific mActb-HaloTag expression. Pups were obtained at P3 by crossing females from one of the FLEx-mActb-HaloTag mouse F0-founder lines with Gfi1-Cre males. Hair cells and stereocilia are stained only in Gfi1-Cre (+) mice (arrow). Stained by JF549 HaloTag-ligand (yellow) and phalloidin (magenta). (C) mActb-HaloTag detected in microvilli (0.5–1 μm) in inner hair cells at P0. Treadmilling seems to have ceased in this microvillus since mActb-HaloTag is mainly expressed at the tip (between arrowheads) but not along the shaft (between open arrowheads). Bars, 5 μm.

mActb-HaloTag can also be induced Tamoxifen-dependent Cre, CAGGCre-ER™ (Figure 4). After approval from the animal facility in NIH, I injected Tamoxifen dissolved in corn oil into the stomoch of neonates at P0 and P1 (Pitulescu et al., 2010; Whitfield et al., 2015). Cochlear sensory epithalia were harvested at P2 or P3 and stained using the JF549-ligand. I also added detergent to the fixative to reduce the signal from soluble mActb-HaloTag. At P2, the mActb-HaloTag was detected mainly at stereocilia tips indicating that treadmilling seems to have ceased at P2.

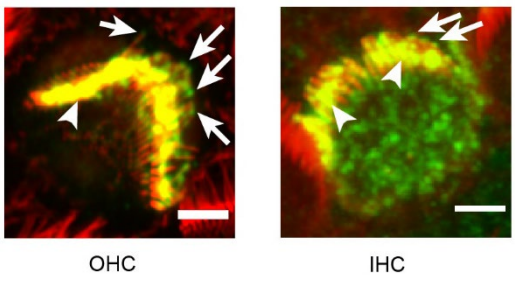

Figure 4: Induction of mActb-HaloTag using Tamoxifen-dependent Cre. mActb-HaloTag is detected at the tips indicating ceased treadmilling (arrowheads and arrows). Injection P0 and P1. Harvest on P3. Bar, 2 μm.

To visualize treadmilling cessation in the earlier developmental stage, I am now developing a protocol to induce mActb-HaloTag expression in a more time-sensitive manner, for example, by using one dose of Tamoxifen and by harvesting cochleae after 24 hours. This protocol will be helpful to visualize F-actin treadmilling more sensitively by reducing the background signal of mActb-HaloTag

directly incorporated to the shaft of F-actin cores. I am also testing induction in explant cultures using 4-OHT to visualize mActb-HaloTag incoporation during the perinatal period, E15–P2, when microvilli morphologically differentiate into stereocilia (Sekerkova et al., 2011).

3. STED super-resolution imaging of microvilli and nascent stereocilia

Imaging of microvilli during the transition to stereocilia (0.5–1 μm) is impossible for conventional confocal microscopes and challenging even for the Airyscan confocal microscope LSM880 available in Dr. Friedman's laboratory (see Figure 3C). For a more precise visualization of microvilli, I started using STED super-resolution microscopy available in Dr. Comb's laboratory in NHLBI. At present, I suceeded in visualizing microvilli in P0 cochleae using phalloidin staining, and I am developing a protocol of dual-channel acquistion to detect mActb-HaloTag incorporated into the microvilli of P0 hair cells. Compared with confocal microscopy including the Airyscan technology, the resolution achieved by STED microscopy is dependent on the fluorescent dyes and the labeling density of imaging targets. In addition, breed-through between channels is more likely to occur as the samples are labeled with more bright dyes and at a higher density. With the time-sensitive induction described in the previous section, I will proceed to the final goal of this study, visualization of actin turnover during the microvilli-to-transition to elucidate the molecular mechanism maintaining the entire architectures stereocilia during a lifetime of a person.

Future directions

Currently, I am developing methods to induce mActb-HaloTag in a more time-sensitive manner and to visualize them using STED super-resolution microscopy. After these methods are established, I will proceed to identification of molecules involved in the treadmilling cessation using transcriptomics of hair cells using single-cell and single-nucleus RNA-seq in a collaboration work with Dr. Mike Hoa's laboratory.

Effect of COVID-19 pandemic

Due to the pandemic of COVID-19, laboratories in NIH was shutdown for about 3 months (March 2020 – June 2020). Experiments including breeding and evaluation of transgenic mice were strictly restricted during this period. The laboratories reopened on 6/23/20 with a 33% limited occupancy. Until vaccination to general staff started around Mar 2021, the occupancy remained 50%, and the access to equipment including the STED microscope was also limited. These restrictions delayed my research schedule, which was not fully recovered even with the 6-month extension period. Currently (Oct 2021), I am allowed to work on site all day under the mask mandatory and social distancing. I am now woking as a visiting fellow under the support of Dr. Friedman to accomplish the goal this study.

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