

Virus-induced expression of retinoic acid inducible gene-I and melanoma differentiation-associated gene 5 in the cochlear sensory epithelium

Yushi Hayashi<sup>a</sup>, Koji Onomoto<sup>b,c</sup>, Ryo Narita<sup>b,d</sup>, Mitsutoshi Yoneyama<sup>b,c</sup>, Hiroki Kato<sup>b,d</sup>, Takayuki Nakagawa<sup>a</sup>, Juichi Ito<sup>a</sup>, Akiko Taura<sup>a</sup>, Takashi Fujita<sup>b,d</sup>\*

a) Department of Otolaryngology and Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

b) Laboratory of Molecular Genetics, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

c) Division of Molecular Immunology, Medical Mycology Research Center, Chiba University, Chiba 260-8673, Japan

d) Laboratory of Molecular Cell Biology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8507, Japan

Corresponding author:

Takashi Fujita

Phone/Fax +81-75-751-4031, tfujita@virus.kyoto-u.ac.jp

Department of Molecular genetics, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

## Abstract

The inner ear has been regarded as an immunoprivileged site because of isolation by the blood-labyrinthine barrier. Several reports have indicated the existence of immune cells in the inner ear, but there are no reports showing immunocompetence of the cochlear tissue. In this report, we examined the potential involvement of retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which are critical for initiating antiviral innate immune responses. We found that RIG-I and MDA5 are expressed in the mouse cochlear sensory epithelium, including Hensen's and Claudius' cells. Ex vivo viral infection using Theiler's murine encephalomyelitis virus revealed that the virus replicates in these cells and that protein levels of RIG-I and MDA5 are up-regulated. Furthermore, the critical antiviral transcription factor, interferon (IFN) regulatory factor-3, is activated in the infected cells as judged by its nuclear translocation and the accumulation of type I IFN transcripts. These results strongly suggest that RIG-I and MDA5 participate in innate antiviral responses in cochlear tissue.

*Keywords:* Innate immunity, retinoic acid inducible gene-I family, interferon, cochlear sensory epithelium, Supporting cells, Hensen's and Claudius' cells

## 1. Introduction

The inner ear has been regarded as an immunoprivileged site like the central nervous system, or the cornea or the retina of the eye because of isolation by the blood-labyrinthine barrier [1-3]. Several studies have recently revealed the presence of immunocompetent cells in the cochlea, the vestibular end organ, and the endolymphatic sac [4-7]. These reports indicate the existence of immune cells in the inner ear tissue, but there are no reports which indicate that the cochlear tissue itself possesses immunocompetence.

Higher animals such as *Homo sapiens* have two kinds of immunocompetence, innate and adaptive immunity, when viruses or bacteria infect the body [8]. Adaptive immunity, specific immune reactions to antigens, is very effective and acts by establishing immunological memory, but it requires education through exposure to antigens, thus requiring a time period of more than one week. In contrast, innate immunity is operative soon after initial infection and plays critical roles in guiding adaptive immunity.

Retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which encode a helicase domain and two tandem repeats of caspase recruitment domain, were identified as a sensor of viral double-stranded (ds) RNA [9-13]. Once RIG-I or MDA5 is activated, the signal is transmitted through adaptor molecules finally resulting in the activation of transcription factors, interferon (IFN) regulatory factor (IRF)-3, -7 and nuclear factor- $\kappa$ B (NF- $\kappa$ B).

IFNs are cytokines which have anti-viral activity. They are classified into type I including IFN  $\alpha$  and IFN  $\beta$ , type II including IFN  $\gamma$  and type III including IFN  $\lambda$ . Type I and III IFNs, which are transcriptionally activated by IRF-3/-7 and NF- $\kappa$ B, provoke innate immune reactions and contribute to triggering adaptive immunity [14-17]. Thus, RIG-I and MDA5 are key molecules in the detection and subsequent eradication of the replicating viral genomes through the production of IFNs

There are some diseases of the cochlea which are possibly related to viral infection such as sudden hearing loss or congenital sensorineural hearing

loss [18-25]. However, there are no reports which clarify the relationship between viral infection and inner ear disease. The aim of this study was to investigate whether the cochlea possesses immunocompetence against viruses through RIG-I and MDA5.

## 2. Materials and Methods

### *2.1 Animals*

ICR mice were purchased from Japan SLC, Hamamatsu, Japan. All experimental procedures were performed in accordance with the NIH Guide for the care and use of laboratory animals. The Animal Research Committee, Graduate School of Medicine, Kyoto University, approved all experimental protocols. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

### *2.2 Preparation of explant culture treatment of the cochlear sensory epithelium*

Postnatal day (P) 2 ICR mice were deeply anesthetized with carbon dioxide and decapitated. Cochleae were dissected out from the heads of mice using watchmaker forceps under a stereomicroscope. Organs of Corti were separated from surrounding tissues and put on cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA). Cultures were incubated at 37°C in a

humidified 5% CO<sub>2</sub> atmosphere with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Inc., St. Louis, MO, USA), supplemented with D-glucose (6 g/l) and penicillin G. To exclude the possibility of effects due to several cytokines contained in the serum, we used serum-free medium in all experiments. All the experiments were started after 24-hours of incubation with the medium to stabilize the explants. The explants were then transferred to the medium containing 1 x 10<sup>6</sup> pfu/ml Theiler's murine encephalomyelitis virus (TMEV). TMEV (GDVII strain) was propagated from viral cDNA and BHK21 cells [26].

### *2.3 Immunohistochemistry*

For sectioned samples, P2 mice cochleae were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 4 h, embedded in an OCT compound (Tissue Tek, Miles Inc., IN), and 10 µm mid-modiolar sections were prepared.

For whole mount immunohistochemistry, samples were fixed at room



temperature (RT) for 15 min in 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4).

Specimens were rinsed with phosphate buffered saline (PBS), incubated in blocking solution at RT (30 minutes in 10% goat serum with 0.2% TritonX-100).

Primary antibodies, rabbit polyclonal anti-RIG-I (1:500), rabbit polyclonal anti-MDA5 (1:500), and rabbit polyclonal anti-IRF-3 (1:500) were described previously [9, 11, 27, 28]. The mouse monoclonal anti-dsRNA (J2 1:900, K1 1:2000) were obtained from English & Scientific Consulting, Hungary. Actin filaments were visualized with Alexa 546 or 633-labeled phalloidin (1:100; Invitrogen).

Primary antibodies were visualized with Alexa-488 or 546-conjugated anti-rabbit or mouse goat IgG (1:1000; Invitrogen). Specimens for which nuclear staining was needed were then incubated in PBS containing 2 mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen).

Fluorescent images were captured with a Leica TCS-SPE confocal

microscope (Leica Microsystems Inc., Wetzlar, Germany).

#### *2.4 Western blotting*

20 explants incubated with or without  $1 \times 10^6$  pfu/ml TMEV for 9 h were lysed with Nonidet P-40 lysis buffer (50mM Tris (pH 8), 150mM NaCl, and 1% Nonidet P-40). The lysate was resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk for 30 min at RT and probed with rabbit polyclonal anti-RIG-I (1:500), rabbit polyclonal anti-MDA5 (1:500) and rabbit polyclonal anti-IRF-3 (1:500) antibodies. Antibody binding was detected with alkaline phosphatase-conjugated anti-rabbit IgG.

#### *2.5 Quantitative RT-PCR (qRT-PCR)*

To evaluate expression of RIG-I, MDA5, IRF-3 and GAPDH in different tissues, total RNA was prepared from ~~cochlea~~ cochlea of P2 ICR mice, brain, kidney, spleen, liver and lung of 6w DBA/2 mice with TRIzol reagent

(Invitrogen). For virus-infected cochlear tissue, total RNA was extracted from three explants incubated with or without  $1 \times 10^6$  pfu/ml TMEV for 9 h using TRIzol. The RNA was amplified by reverse transcription-PCR with the ABI StepOnePlus Real-Time PCR System (Applied Biosystems). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. We used commercial TaqMan Fast Advanced Master Mix and TaqMan primer-probe sets (Applied Biosystems) for mouse RIG-I (Ddx58), MDA5 (Ifih1), Irf3, Gapdh, IFN  $\alpha$ 4 (Ifna4) and IFN  $\beta$ 1 (Ifnb1). As an internal control for the comparative threshold cycle methods, a primer-probe set for eukaryotic 18 s rRNA (Applied Biosystems) was used. The results were normalized to the abundance of internal control.

## *2.6 Statistical analysis*

Statistical analyses were performed using the unpaired *t* test with *P* values below 0.05 considered statistically significant.

### 3. Results

#### *3.1 Hensen's and Claudius' cells expressed RIG-I and MDA5, but their expression was absent in cochlear hair cells.*

The expression of RIG-I and MDA5 was examined in the uninfected cochlear sensory epithelium by immunostaining (Fig. 1). For this purpose we used antibodies that specifically detect mouse RIG-I and MDA5 by immunostaining [11, 28]. RIG-I and MDA5 were expressed weakly in the outside of the outer hair cell area (excluding outer hair cells, OHC and inner hair cells, IHC) as revealed by whole mount views (Fig. 1 A, B). Cryosection revealed that these cells with RIG-I and MDA5 expression corresponded to Hensen's and Claudius' cells and cochlear hair cells (OHC and IHC), while Deiters' cells were negative (Fig. 1 C, D). RIG-I and MDA5 localized mainly in the cytoplasm in these cells.

Next, we examined mRNA expression in the mouse tissues by qRT-PCR. Messenger RNA levels of RIG-I, MDA5, IRF-3 and housekeeping GAPDH were quantified in cochlear and other tissues (Fig. 2). Expression of RIG-I

and MDA5 was detectable in these tissues except expression of RIG-I and IRF-3 was very low in brain. Whereas expression of RIG-I and MDA5 was relatively high in kidney, spleen, liver and lung, where antiviral innate immunity is operative [29]. Interestingly, cochlea expresses RIG-I transcript at higher levels compared to brain. On the other hand, MDA5 expression in cochlea is comparable to that in brain. It should be noted that RIG-I and MDA5 are expressed in minor fraction of cochlear cells (Fig. 1). Expression of IRF-3 mRNA was ubiquitous in these tissues and consistent with a notion that IRF-3 protein level is not regulated at the level of transcription.

### *3.2 The expression of RIG-I and MDA5 was induced after viral infection in the cochlea.*

To evaluate antiviral response in cochlea, we used TMEV because this virus induces encephalomyelitis, therefore may have an affinity to cochlear sensory epithelia. The cochlear culture was infected with TMEV and

expression of RIG-I and MDA5 was examined (Fig. 3). After infection for 9 hr, dsRNA became detectable in the area of Hensen's and Claudius' cells (Fig. 3 A, C), indicating that these supporting cells are highly susceptible to TMEV. In these dsRNA- positive cells, the expression of RIG-I and MDA5 was dramatically increased as compared with uninfected cells (Fig. 3 B, D, Fig. 1 A, B). On the other hand, there were no dsRNA-positive cells in the hair cell layer (OHC and IHC) and their hair bundles were morphologically intact.

To examine RIG-I and MDA5 proteins, we performed Western blotting of the cochlea using specific antibodies. TMEV infection resulted in augmentation of RIG-I and MDA5 proteins (Fig. 4). This is consistent with the notion that expression of RIG-I-like receptor (RLR) is inducible by viral infection and IFN treatment [9-11, 30].

### *3.3 Nuclear translocation of IRF-3 was induced by TMEV in supporting cells.*

A transcript of IRF-3, a critical transcription factor for the activation of IFN genes, was expressed in the whole cochlea (Fig. 2) and IRF-3 protein

was accumulated in the cochlea. TMEV infection did not alter the overall protein level of IRF-3 (Fig. 4). Results of histochemical analysis showed that IRF-3 was detectable in Hensen's and Claudius' cells (Fig. 5 A, B), in which RIG-I and MDA5 are expressed. Interestingly, cellular localization of IRF-3 was dramatically changed by TMEV infection: virus-infected cells (dsRNA positive) exhibited nuclear accumulation of IRF-3 (Fig. 5), suggesting that TMEV infection activated IRF-3 in the cochlea.

#### *3.4 Activation of IFN $\alpha$ 4 and IFN $\beta$ 1 genes by TMEV infection in the cochlea.*

To clarify if TMEV infection induces type I IFN in the cochlea, total RNA was extracted from uninfected and infected cochleae, and subjected to qRT-PCR (Fig. 6). In the explants without TMEV infection, there was very low transcription of IFN  $\alpha$ 4 and IFN  $\beta$ 1. However, TMEV infection induced high levels of IFN  $\alpha$ 4 and IFN  $\beta$ 1 gene expression (Fig. 6), suggesting that the cochlea recognized viral infection and triggered an antiviral program.

#### 4. Discussion

We report here that RIG-I and MDA5 are expressed in cochlear supporting cells, but not in neuronal hair cells by immunostaining (Fig. 1 and 3), and Western blotting (Fig. 4). It is reported that glial fibrillary acidic protein, a marker of glial cells is expressed in cochlear supporting cells [31]. This finding suggests that supporting cells of the inner ear are functionally similar to glial cells and may play roles similar to those of astrocytes. Glial cells are reported to detect viral pathogens in central nervous system and participate protective immune responses through signal cascades of RIG-I family [32, 33]. These findings suggest an interesting possibility that RLR molecules in cochlear supporting cells play important roles in the detection of viral pathogens in the inner ear and protects cochlear hair cells, which are essential for hearing. Our analysis revealed cell-specific expression of these sensors in the supporting cells: Hensen's and Claudius' cells, but not Deiters' cells, which are distinctly differentiated cell types [34]. Although the biological significance of this cell type-specific expression is not clear, ex vivo



infection of TMEV revealed that intracellular accumulation of viral dsRNA is correlated with increased RIG-I and MDA5 levels and nuclear translocation of IRF-3, strongly suggesting that the viral infection is sensed by either of these RNA sensors and an antiviral response is triggered. Because TMEV belongs to *Picornaviridae*, we speculate MDA5 played a major role in our ex vivo experiment [12, 35]. This is the first report that the cochlear sensory epithelium expresses the RIG-I family and produces IFNs.

Harris et al. reported that systemic adaptive immunity has the potential to affect the inner ear [36], and Wackym reported that herpes viral DNA was detected in temporal bone sections of Ramsay Hunt syndrome [37]. These reports strongly suggest the homeostatic and pathological role of RLR in the inner ear.

It was reported that administration of IFN  $\alpha$  is effective to treat sudden hearing loss patients [38]. In contrast, long-term IFN treatment for viral hepatitis is suggested to cause sensory neural hearing loss [39]. In summary, IFNs may have dual roles in the cochlear sensory epithelium:

protection and damage to cochlear hair cells. Thus, analyses of the innate immune responses in the inner ear of knock out mice, in which genes of RLRs or downstream signal molecules were deleted, will provide insight into inner ear-related disorders such as sudden hearing loss.

## Acknowledgements

We thank Dr. R.P. Roos (UC Irvine), Dr. Y. Ohara (Kanazawa Medical Univ.) and Dr. S. Koike (Tokyo Metropolitan Inst. Med. Sci.) for TMEV GVII clone.

This research was supported by the following grants.

The Ministry of Education, Culture, Sports, Science and Technology in Japan (Innovative Areas “Infection competency” (No.24115004), Scientific Research “A”(23249023)) (<http://www.mext.go.jp/english/>), the Ministry of Health, Labour and Welfare of Japan (<http://www.mhlw.go.jp/english/index.html>), the Uehara Memorial Foundation (<http://www.ueharazaidan.com/>), the Mochida Memorial Foundation for Medical and Pharmaceutical Research (<http://www.mochida.co.jp/zaidan/>), the Takeda Science Foundation (<http://www.takeda-sci.or.jp/index.html>), the Naito Foundation (<http://www.naito-f.or.jp/>), and Nippon Boehringer Ingelheim (<http://www.boehringer-ingelheim.co.jp/com/Home/index.jsp>).

## References

- [1] Harris JP, Ryan AF, Fundamental immune mechanisms of the brain and inner ear, *Otolaryngol Head Neck Surg* 112 (1995) 639-653.
- [2] Hughes RA, Gray IA, Gregson NA, Kadlubowski M, Kennedy M, Leibowitz S, Thompson H, Immune responses to myelin antigens in Guillain-Barré syndrome, *J Neuroimmunol* 6 (1984) 303-312.
- [3] Barker CF, Billingham RE, Immunologically privileged sites, *Adv Immunol* 25 (1977) 1-54.
- [4] Lang H, Ebihara Y, Schmiedt RA, Minamiguchi H, Zhou D, Smythe N, Liu L, Ogawa M, Schulte BA, Contribution of bone marrow hematopoietic stem cells to adult mouse inner ear: mesenchymal cells and fibrocytes, *J Comp Neurol* 496 (2006) 187-201.
- [5] Okano T, Nakagawa T, Kita T, Kada S, Yoshimoto M, Nakahata T, Ito J, Bone marrow-derived cells expressing Iba1 are constitutively present as resident tissue macrophages in the mouse cochlea, *J Neurosci Res* 86 (2008) 1758-1767.
- [6] Okano T, Nakagawa T, Ito J, Distribution of bone marrow-derived cells in the vestibular end organs and the endolymphatic sac, *Acta Otolaryngol Suppl* (2010) 88-94.
- [7] Sato E, Shick HE, Ransohoff RM, Hirose K, Repopulation of cochlear macrophages in murine hematopoietic progenitor cell chimeras: the role of CX3CR1, *J Comp Neurol* 506 (2008) 930-942.
- [8] Medzhitov R, Janeway CA, Innate immune recognition and control of adaptive immune responses, *Semin Immunol* 10 (1998) 351-353.
- [9] Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T, The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses, *Nat Immunol* 5 (2004) 730-737.
- [10] Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M, Jr., Akira S, Yonehara S, Kato A, Fujita T, Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity, *J Immunol* 175 (2005) 2851-2858.
- [11] Onomoto K, Jogi M, Yoo JS, Narita R, Morimoto S, Takemura A, Sambhara S, Kawaguchi A, Osari S, Nagata K, Matsumiya T, Namiki H, Yoneyama M, Fujita T, Critical role of an antiviral stress granule containing RIG-I and PKR in viral detection and innate immunity, *PLoS One* 7 (2012) e43031.

- [12] Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S, Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses, *Nature* 441 (2006) 101-105.
- [13] Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S, Cell type-specific involvement of RIG-I in antiviral response, *Immunity* 23 (2005) 19-28.
- [14] Le Bon A, Tough DF, Links between innate and adaptive immunity via type I interferon, *Curr Opin Immunol* 14 (2002) 432-436.
- [15] Samuel CE, Antiviral actions of interferons, *Clin Microbiol Rev* 14 (2001) 778-809, table of contents.
- [16] Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD, How cells respond to interferons, *Annu Rev Biochem* 67 (1998) 227-264.
- [17] Onoguchi K, Yoneyama M, Takemura A, Akira S, Taniguchi T, Namiki H, Fujita T, Viral infections activate types I and III interferon genes through a common mechanism, *J Biol Chem* 282 (2007) 7576-7581.
- [18] Sugiura S, Yoshikawa T, Nishiyama Y, Morishita Y, Sato E, Beppu R, Hattori T, Nakashima T, Detection of herpesvirus DNAs in perilymph obtained from patients with sensorineural hearing loss by real-time polymerase chain reaction, *Laryngoscope* 114 (2004) 2235-2238.
- [19] Wilson WR, Byl FM, Laird N, The efficacy of steroids in the treatment of idiopathic sudden hearing loss. A double-blind clinical study, *Arch Otolaryngol* 106 (1980) 772-776.
- [20] Khetarpal U, Nadol JB, Glynn RJ, Idiopathic sudden sensorineural hearing loss and postnatal viral labyrinthitis: a statistical comparison of temporal bone findings, *Ann Otol Rhinol Laryngol* 99 (1990) 969-976.
- [21] Schuknecht HF, Benitez J, Beekhuis J, Igarashi M, Singleton G, Ruedi L, The pathology of sudden deafness, *Laryngoscope* 72 (1962) 1142-1157.
- [22] Wilson WR, Veltri RW, Laird N, Sprinkle PM, Viral and epidemiologic studies of idiopathic sudden hearing loss, *Otolaryngol Head Neck Surg* 91 (1983) 653-658.
- [23] Sugiura S, Yoshikawa T, Nishiyama Y, Morishita Y, Sato E, Hattori T, Nakashima T, Detection of human cytomegalovirus DNA in perilymph of patients with sensorineural hearing loss using real-time PCR, *J Med Virol* 69 (2003) 72-75.

- [24] Mentel R, Kaftan H, Wegner U, Reissmann A, Gürtler L, Are enterovirus infections a co-factor in sudden hearing loss?, *J Med Virol* 72 (2004) 625-629.
- [25] Di Nardo W, Cattani P, Lopizzo T, Cantore I, Marchese MR, Marchetti S, Scorpecci A, Giannantonio S, Parrilla C, Cianfrone F, Fadda G, Paludetti G, Multiple viral genome search in endolabyrinthic fluids of profoundly deaf patients: possible cytomegalovirus intracochlear reactivation, *Audiol Neurootol* 14 (2009) 290-295.
- [26] Fu JL, Stein S, Rosenstein L, Bodwell T, Routbort M, Semler BL, Roos RP, Neurovirulence determinants of genetically engineered Theiler viruses, *Proc Natl Acad Sci U S A* 87 (1990) 4125-4129.
- [27] Iwamura T, Yoneyama M, Yamaguchi K, Suhara W, Mori W, Shiota K, Okabe Y, Namiki H, Fujita T, Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways, *Genes Cells* 6 (2001) 375-388.
- [28] Onoguchi K, Onomoto K, Takamatsu S, Jogi M, Takemura A, Morimoto S, Julkunen I, Namiki H, Yoneyama M, Fujita T, Virus-infection or 5'ppp-RNA activates antiviral signal through redistribution of IPS-1 mediated by MFN1, *PLoS Pathog* 6 (2010) e1001012.
- [29] Ida-Hosonuma M, Iwasaki T, Yoshikawa T, Nagata N, Sato Y, Sata T, Yoneyama M, Fujita T, Taya C, Yonekawa H, Koike S, The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus, *J Virol* 79 (2005) 4460-4469.
- [30] Barber MR, Aldridge JR, Webster RG, Magor KE, Association of RIG-I with innate immunity of ducks to influenza, *Proc Natl Acad Sci U S A* 107 (2010) 5913-5918.
- [31] Rio C, Dikkes P, Liberman MC, Corfas G, Glial fibrillary acidic protein expression and promoter activity in the inner ear of developing and adult mice, *J Comp Neurol* 442 (2002) 156-162.
- [32] Furr SR, Moerdyk-Schauwecker M, Grdzlishvili VZ, Marriott I, RIG-I mediates nonsegmented negative-sense RNA virus-induced inflammatory immune responses of primary human astrocytes, *Glia* 58 (2010) 1620-1629.
- [33] Furr SR, Chauhan VS, Sterka D, Grdzlishvili V, Marriott I, Characterization of retinoic acid-inducible gene-I expression in primary murine glia following exposure to vesicular stomatitis virus, *J Neurovirol* 14 (2008) 503-513.
- [34] Buckiová D, Syka J, Calbindin and S100 protein expression in the developing inner ear in mice, *J Comp Neurol* 513 (2009) 469-482.

- [35] Jin YH, Kim SJ, So EY, Meng L, Colonna M, Kim BS, Melanoma differentiation-associated gene 5 is critical for protection against Theiler's virus-induced demyelinating disease, *J Virol* 86 (2012) 1531-1543.
- [36] Harris JP, Woolf NK, Ryan AF, Butler DM, Richman DD, Immunologic and electrophysiological response to cytomegaloviral inner ear infection in the guinea pig, *J Infect Dis* 150 (1984) 523-530.
- [37] Wackym PA, Molecular temporal bone pathology: II. Ramsay Hunt syndrome (herpes zoster oticus), *Laryngoscope* 107 (1997) 1165-1175.
- [38] Kanemaru S, Fukushima H, Nakamura H, Tamaki H, Fukuyama Y, Tamura Y, Alpha-Interferon for the treatment of idiopathic sudden sensorineural hearing loss, *Eur Arch Otorhinolaryngol* 254 (1997) 158-162.
- [39] Kanda Y, Shigeno K, Kinoshita N, Nakao K, Yano M, Matsuo H, Sudden hearing loss associated with interferon, *Lancet* 343 (1994) 1134-1135.

## Figure legends

### Fig. 1

Expression of RIG-I and MDA5 in the cochlear sensory epithelium.

Whole mounts of the cochlear sensory epithelium (A, B), and cryosection of the cochlear sensory epithelium (C, D), were stained for RIG-I, MDA5 (green) and actin (phalloidin, red) as indicated. The sections were counterstained by DAPI to visualize nuclei. OHC: outer hair cells. IHC: inner hair cells. Scale bars: 25  $\mu$ m.

### Fig. 2

Expression of mRNA encoding RIG-I, MDA5, IRF-3 and GAPDH in mouse tissues. RNA extracted from mouse tissues was subjected to qRT-PCR analyses as described in Materials and Methods. Relative mRNA copy number, cochlea mRNA level as 1.00 is shown.

### Fig. 3



Change in the RIG-I and MDA5 expression patterns in the cochlear sensory epithelium induced by TMEV infection. The cochlear culture was infected with TMEV for 9 hr and stained for dsRNA (A-D, red), phalloidin (A-D, white), DAPI (A, C, blue), RIG-I (B, green) or MDA5 (D, green). Scale bar: 25  $\mu\text{m}$ .

Fig. 4

The change in RIG-I, MDA5 and IRF-3 proteins level caused by TMEV infection. When infected by TMEV, the amount of RIG-I and MDA5 protein increased, which indicated that TMEV infection induces expression of RIG-I and MDA5. However, the protein level of IRF-3 was not changed so much, which indicated that TMEV infection does not induce the expression level of IRF-3 dramatically.

Fig. 5

The expression pattern of IRF-3 in the cochlear sensory epithelium.

Uninfected (A, B), and TMEV-infected (C, D) cochlear tissues were examined for IRF-3, phalloidin, dsRNA and DNA (DAPI) as indicated. C and D are the same whole mounts with different filters. Nuclei and IRF-3 shown by blue (C) and green (D), respectively, are indicated by arrowheads. Scale bars: 25  $\mu\text{m}$ .

Fig. 6

TMEV induces expression of IFN  $\alpha$ 4 and IFN  $\beta$ 1 genes in the cochlear sensory epithelium. Total RNA was extracted from uninfected (TMEV(-)) and infected (TMEV(+)) cochlear tissues and mRNA copies of IFN  $\alpha$ 4 (A) and IFN  $\beta$ 1 (B) were determined by qRT-PCR. TMEV infection enhanced the IFN  $\alpha$ 4 and IFN  $\beta$ 1 mRNA copy numbers by  $1.0322 \times 10^6$  times and  $1.0336 \times 10^6$  times, respectively. Bars represent standard errors. \* $P < 0.05$ ; \*\* $P < 0.01$ ; by Student's t-test.

Fig. 1

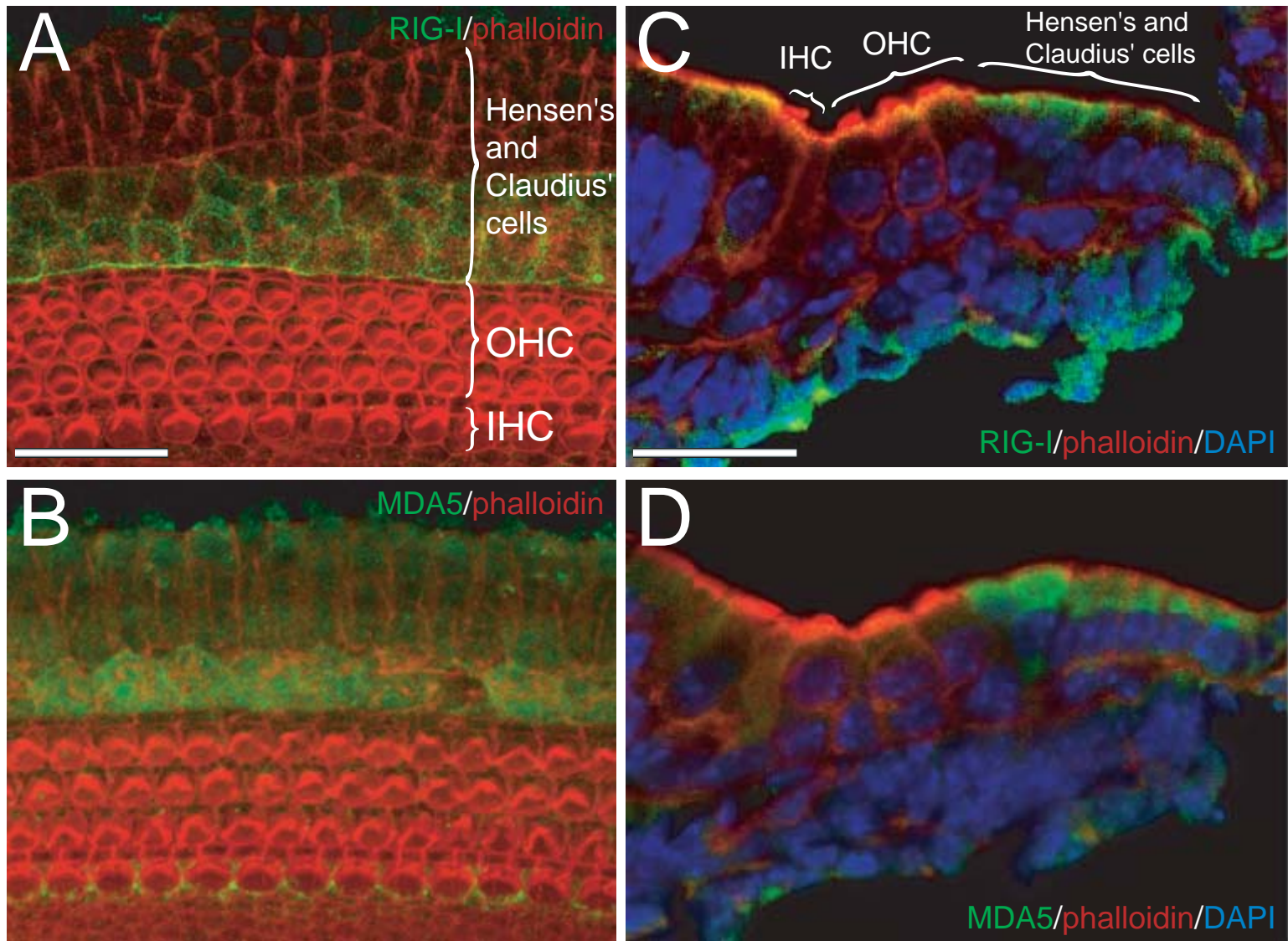


Fig. 2

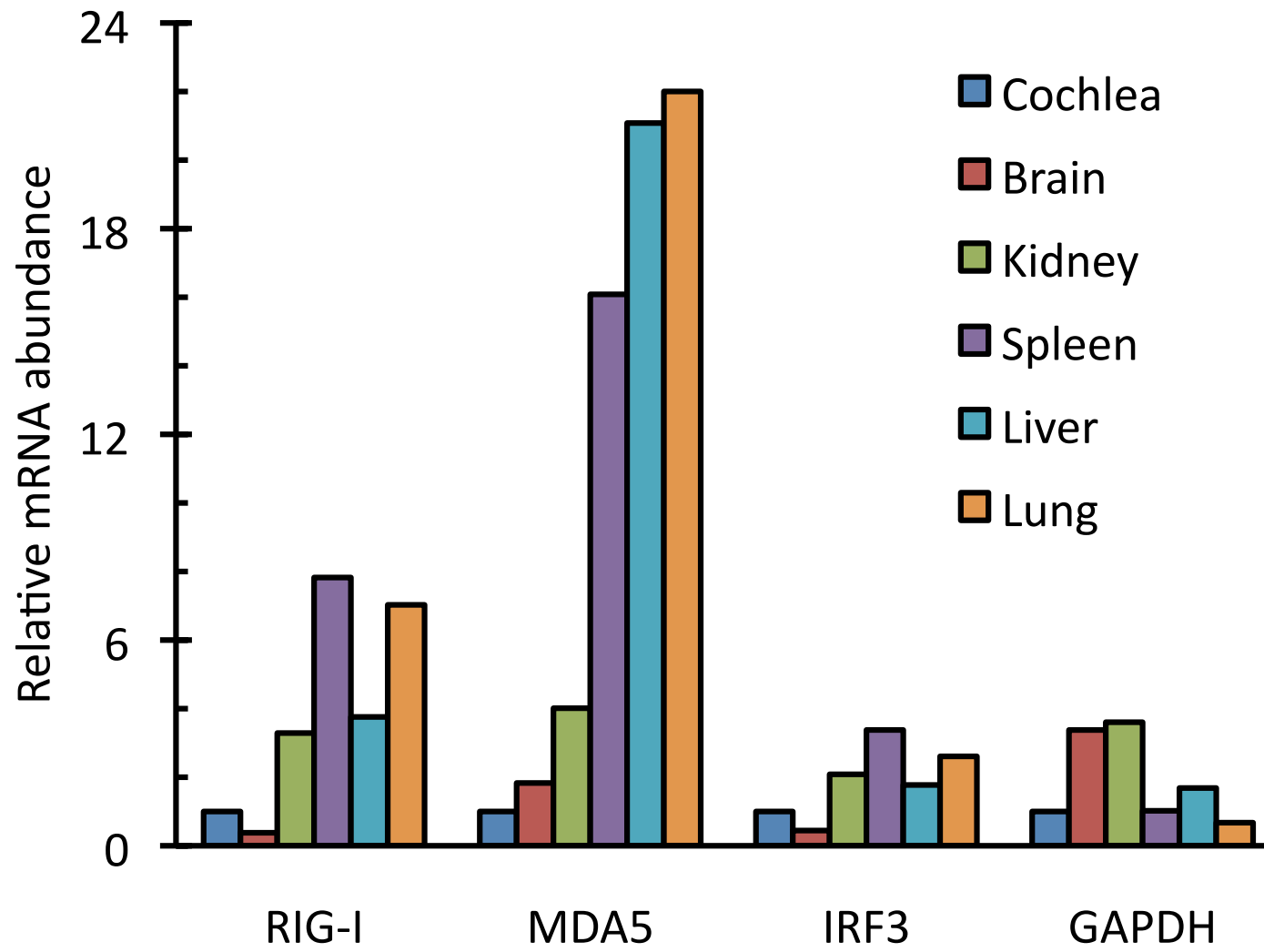


Fig. 3

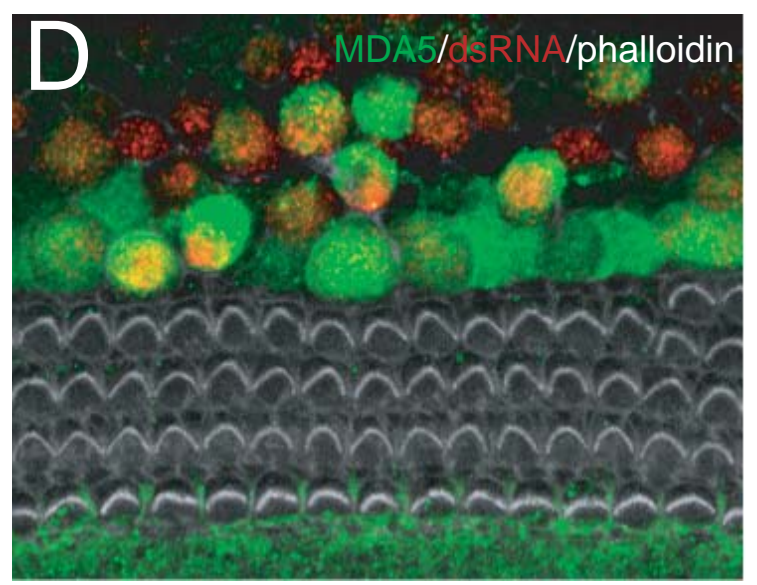
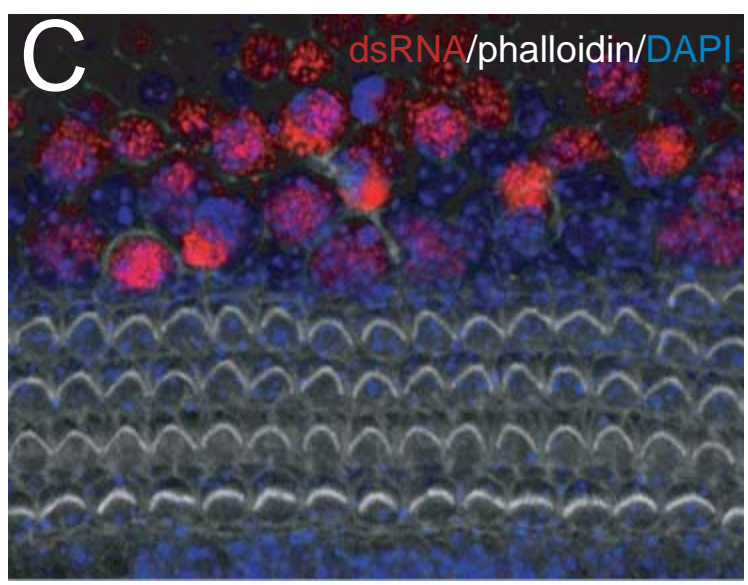
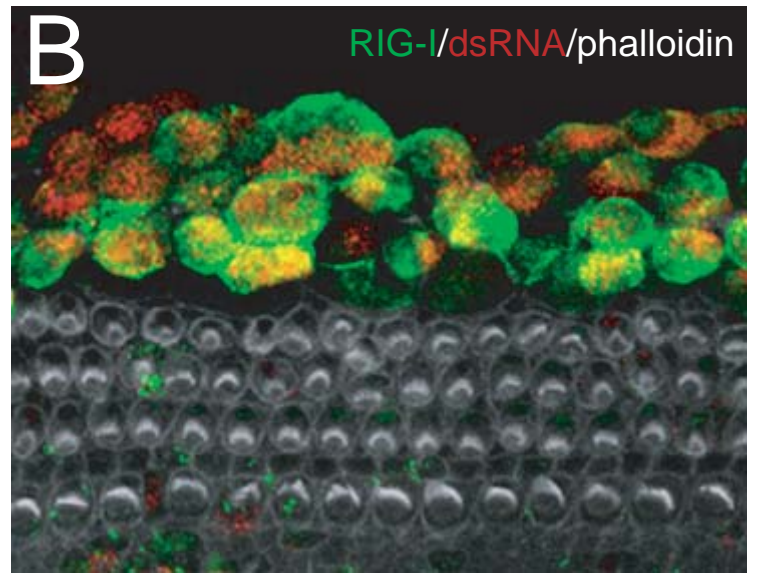
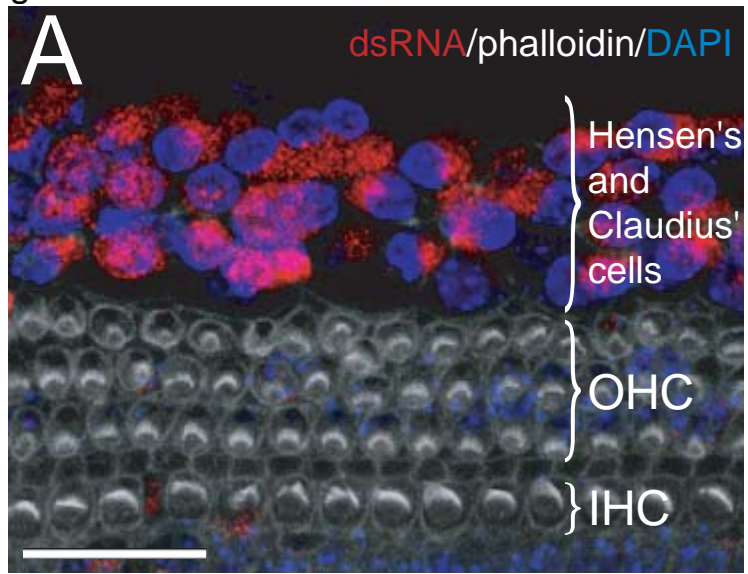


Fig. 4

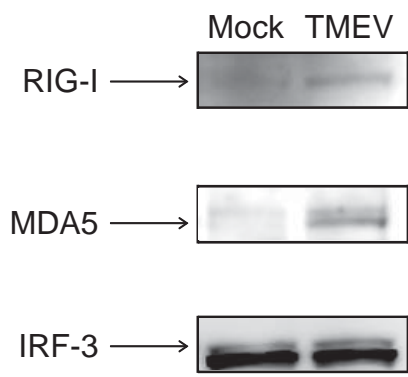




Fig. 5

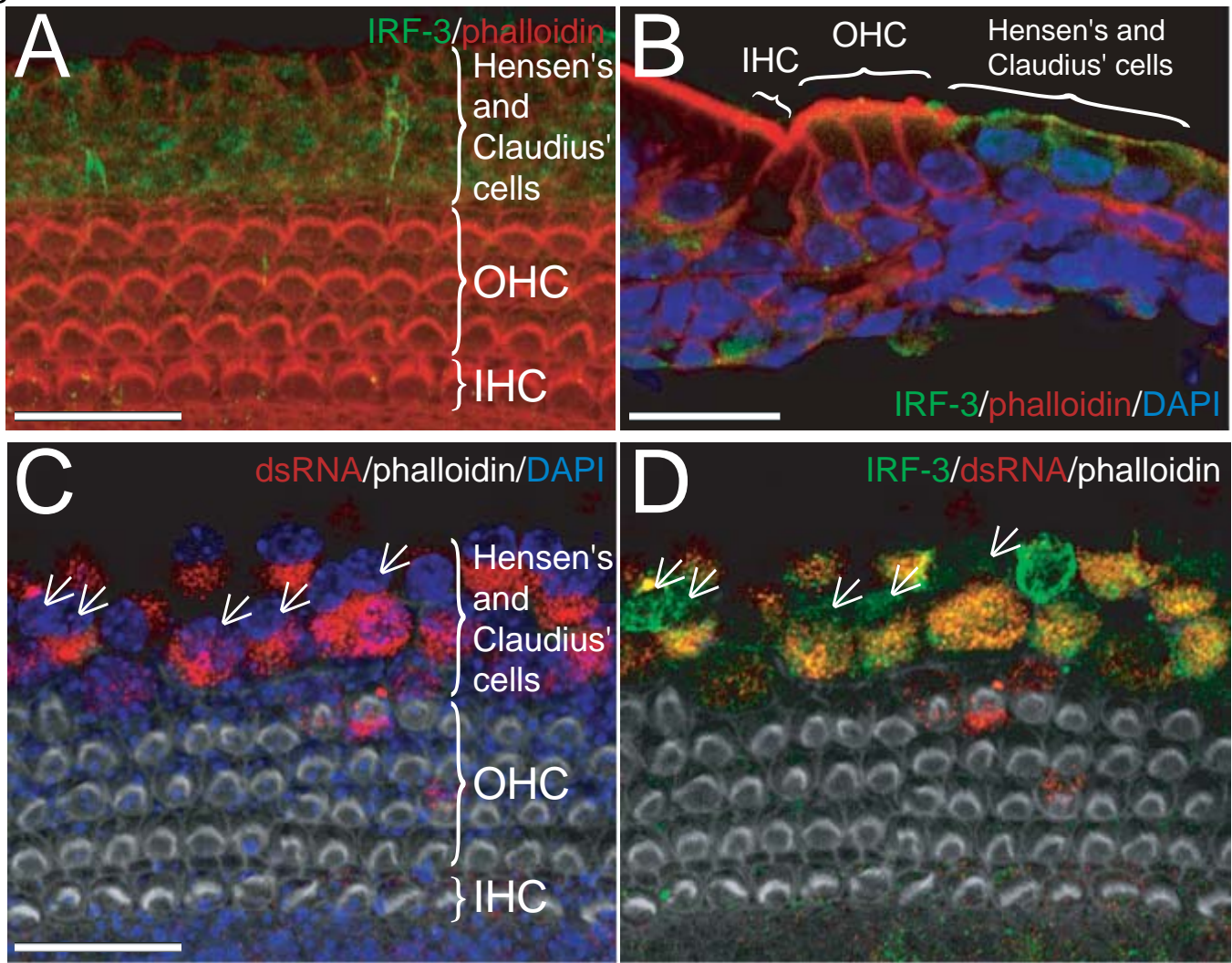


Fig. 6

