Expression of *Ace2*, *Tmprss2*, and *Furin* in mouse ear tissue

Tsukasa Uranaka, M.D., Akinori Kashio M.D., Ph.D., Rumi Ueha, M.D., Ph.D.; Taku Sato, M.D., Han Bing, M.D., Gao Ying, M.D., Makoto Kinoshita M.D., Ph.D., Kenji Kondo, M.D., Ph.D.; Tatsuya Yamasoba M.D., Ph.D.

Department of Otolaryngology and Head and Neck Surgery, Faculty of Medicine, the University of Tokyo, Tokyo, Japan

Key words: SARS-CoV-2, COVID-19, ACE2, TMPRSS2, Furin, Temporal Bone

Correspondence to: Tatsuya Yamasoba, M.D., Ph.D.
Department of Otolaryngology and Head and Neck Surgery, Faculty of Medicine, the University of Tokyo, Tokyo, Japan, 113-8655
E-mail: tyamasoba-tky@umin.ac.jp
Tel: +81-3-3815-5411, Fax: +81-3-3814-9486

Author contributions

T.U developed the concept and performed the experiments, and wrote the draft of the manuscript. K. A., R.U., T.S., H.B., G.Y., and M.K. performed the experiments. K.K reviewed the manuscript. T.Y. developed the concept, designed the experiment, and wrote the draft of the manuscript. All authors contributed to interpretation of the data and writing of the manuscript.

Funding

This work was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (A) [grant number 20H00546].

Conflict of Interest Statement

We declare no competing interests.

Abstract

Objectives: Intracellular entry of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) depends on the interaction between its spike protein to a cellular receptor named angiotensin-converting enzyme 2 (ACE2) and depends on Furin-mediated spike 23 protein cleavage and spike protein priming by host cell proteases including 24 transmembrane protease serine 2 (TMPRSS2). *Tmprss1*, *Tmprss3*, and *Tmprss5* are expressed in the spiral ganglion neurons and the organ of Corti in the inner ear; however, *Ace2*, *Tmprss2*, and *Furin* expression profiles in the middle ear remain unclear. Therefore, this study aimed to analyze *Ace2*, *Tmprss2*, and *Furin* expression in the middle and inner ear of mice.

Study Design: Animal research.

Setting: Department of Otolaryngology and Head and Neck Surgery, University of Tokyo.

Methods: We performed immunohistochemical analysis to examine the distribution of Ace2, Tmprss2, and Furin in the eustachian tube, middle ear space, and cochlea of mice.

Results: Ace2 was expressed in the cytoplasm in the middle ear epithelium, eustachian

tube epithelium, stria vascularis, and spiral ganglion. Tmprss2 and Furin were widely expressed in the middle ear spaces and the cochlea.

Conclusion: Co-expression of Ace2, Tmprss2, and Furin in the middle ear indicates that the middle ear is susceptible to SARS-CoV-2 infections, thus warranting the use of personal protective equipment during mastoidectomy for coronavirus disease (COVID-19) patients.

Key words: SARS-CoV-2, COVID-19, ACE2, TMPRSS2, Furin, Temporal Bone

Introduction

The recent coronavirus disease (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) poses a serious health concerns. SARS-CoV-2 generally infects the upper respiratory tract and then spreads to various organs. The clinical symptoms of COVID-19 patients include fever, cough, sore throat, fatigue, and loss or decline of smell (anosmia or hyposmia).[1-4] The Eustachian tube is the conduit between the middle ear space and upper respiratory tract through which,

SARS-CoV-2 can spread into the middle ear spaces. Moreover, other human coronaviruses have been identified in middle ear fluid in cases of acute otitis media or otitis media with effusion.[5-7] Two studies have reported the occurrence of acute otitis media[8] and sensorineural hearing loss[9] among COVID-19 patients. Another study carried out pure tone audiometry and transitory evoked otoacoustic emission (TEOAE) analysis for 20 asymptomatic patients aged 20–50 years positive for COVID-19 on reverse transcription-PCR (RT-PCR) testing and reported that high-frequency pure-tone thresholds and TEOAE amplitudes were significantly worse among the COVID-19 patients than among 20 healthy controls.[10]

Intracellular entry of SARS-CoV-2 depends on the interaction between viral spike proteins and a cellular receptor, angiotensin-converting enzyme 2 (Ace2)[11-13], Furin-mediated Spike cleavage[13-15], and Spike protein priming by host cell proteases including transmembrane protease serine 2 (Tmprss2).[13,16] Thus, Ace2, Tmprss2, and Furin upregulation enhances intracellular SARS-CoV-2 entry, thus resulting in clinical symptoms. Previous studies have reported 8 *Tmprss* genes including *Tmprss2* in the inner ear through RT-PCR analysis, although immunohistochemical analysis revealed

77 the expression of only Tmprss1/3/5 in the inner ear, indicating that Tmprss1/3/5 are
78 expressed in the spiral ganglion neurons and Tmprss3 is also expressed in the organ of
79 Corti.[17] To our knowledge, Ace2, Tmprss2, and Furin expression in the middle ear
80 spaces and Ace2 and Furin expression in the inner ear have not been reported.

81 This study aimed to investigate the mechanism underlying middle ear infection and
82 sensorineural hearing loss in COVID-19, by assessing *Ace2*, *Tmprss2*, and *Furin*
83 expression in the middle and inner ear tissues of mice.

84

85 **Materials and Methods**

86

87 *Experimental samples*

88 Tissue samples were obtained from the mice used in our previous studies because
89 the purchase of new animals has been prohibited in our facility owing to the COVID-19
90 pandemic. Tissue samples were obtained from normal 11-week-old male ICR mice, and
91 paraffin-embedded tissue samples including the middle ear spaces, Eustachian tube area,
92 and cochlea were used (Figure 1, 2). Normal tissue morphology in these regions was

93 confirmed through hematoxylin and eosin staining by a qualified pathologist and by
94 otolaryngologists. All experiments were conducted in accordance with institutional
95 guidelines and with the approval of the Animal Care and Use Committee of the
96 University of Tokyo (No. P18-015)
97
98 *Histological analyses*
99 Immunohistochemical staining was performed for Ace2 and Tmprss2.
100 Four-micrometer-thick serial paraffin-embedded sections were deparaffinized in xylene
101 and dehydrated in ethanol and then treated with 3% H2O2 to block endogenous
102 peroxidase activity and incubated with Blocking One (Nacalai Tesque, 34 Kyoto, Japan)
103 to block non-specific immunoglobulin binding prior to immunostaining. After antigen
104 activation, the tissue sections were probed with primary anti-Ace2 (1:300 dilution;
105 rabbit monoclonal, Abcam, ab108252; Cambridge, UK), anti-Tmprss2 (1:1000 dilution;
106 rabbit monoclonal, Abcam, ab92323; Cambridge, UK), and anti-Furin (1:100 dilution;
107 rabbit monoclonal, 1 Abcam, ab183495; Cambridge, UK) antibodies, followed by
108 probing with appropriate peroxidase-conjugated secondary antibodies and a

diaminobenzidine substrate. Images of all sections were captured using a digital microscope camera (Keyence BZ-X700) with 4×, 10×, and 40× objective lenses.

Results

Ace2, Tmprss2, and Furin were detected in the mucosal epithelium of the Eustachian tube and middle ear spaces and in the cochlea, although their expression pattern varied among tissues. Furthermore, Ace2, Tmprss2, and Furin were co-expressed in the mucosal epithelium of the Eustachian tube and the middle ear spaces, the stria vascularis, and spiral ganglion cells.

Tmprss2 and Furin were expressed in the mucosal epithelial cells in the middle ear spaces; however, Ace2 was only slightly expressed in the cytoplasm of the epithelial cells (Fig.1A). Furthermore, Ace2 and Tmprss2 were expressed in the nuclei of epithelial cells. Ace2, Tmprss2, and Furin expression profiles in the Eustachian tube were almost identical to those in the middle ear spaces (Fig.1B).

In the cochlea, Ace2 was expressed in the nuclei of the hair cells and supporting cells in the organ of Corti, marginal, intermediate, and basal cells in the stria vascularis,

fibrocytes of the spiral ligament, and spiral ganglion cells. Furthermore, Ace2 was slightly expressed in the cytoplasm of strial cells and spiral ganglion cells. Tmprss2 was diffusely strongly expressed in the nuclei and cytoplasm in the organ of Corti, stria vascularis, spiral ligament, and spiral ganglion cells, being greater in the nucleus than in the cytoplasm. Furin was diffusely expressed in the cytoplasm, but not in the nucleus, in the organ of Corti, stria vascularis, spiral ligament, and spiral ganglion cells (Fig.2).

Discussion

This immunohistochemical study shows that Ace2, Tmprss2, and Furin are co-expressed in the mucosal epithelium of the Eustachian tube and middle ear spaces and the organ of Corti, lateral wall, and spiral ganglion cells in the cochlea. In both middle ear tissues and the cochlea, Ace2 is primarily expressed in the nucleus, while Tmprss2 is expressed in the nucleus and cytoplasm, and Furin was expressed primarily in the cytoplasm. These results suggest that middle ear spaces are highly susceptible to

141 SARS-CoV-2 infections spreading from the upper respiratory tract through the
142 Eustachian tube, indicating the possibility of severe sensorineural hearing loss upon
143 intracellular entry of SARS-CoV-2 in the cochlea.

144 Herein, we used ICR mice to determine the distribution of Ace2, Tmprss2, and
145 Furin in the ear tissues. We could not obtain human ear tissue samples for
146 immunostaining; hence, the expression patterns of these proteins in human ear tissues
147 might differ from those of the present mouse ear tissues. However, we previously
148 compared the Ace2, Tmprss2, and Furin expression patterns between human and mouse
149 nasal tissues and found that their expression patterns were identical.[18]

150 Mastoidectomy is required during major ear surgeries including cochlear
151 implantation and middle ear surgery for extensive cholesteatoma. Mastoidectomy with a
152 high-speed drill generates massive aerosols.[19] Previously, cadaveric simulation of
153 otological procedures during mastoidectomy revealed gross contamination 3 to 6 feet
154 away in all cardinal directions and more significantly on the left side, corresponding to
155 the direction of drill rotation.[20] SARS-CoV-2 would substantially infect the middle ear
156 spaces because of the co-expression of Aces, Tmprss2, and Furin. Therefore, high levels

of personal protective equipment are strongly recommended during mastoidectomy for COVID-19 patients or all mastoidectomies during the COVID-19 pandemic.

The cochlea is isolated and thus is generally resistant to the infection. A recent systemic review on the audio-vestibular symptoms of coronavirus infections found no records of audio-vestibular symptoms reported during previous coronaviral diseases including SARS and Middle East respiratory syndrome.[21] However, Ace2, Tmprss2, and Furin were co-expressed in the organ of Corti, lateral wall, and spiral ganglion cells in the cochlea. Thus, similar to other viral infections including mumps, when SARS-CoV-2 enters the cochlea, it can cause severe labyrinthitis, resulting in marked hearing loss.

Conclusions

Ace2, Tmprss2, and Furin are co-expressed in the mucosal epithelium of the Eustachian tube and middle ear spaces and the organ of Corti, lateral wall, and spiral ganglion cells in the cochlea in mice. ACE2 is primarily expressed in the nucleus, Tmprss2 is expressed in the nucleus and cytoplasm, and Furin is primarily expressed in the cytoplasm. These results indicate that middle ear spaces are highly susceptible to the

SARS-CoV-2 infection, thus warranting the extensive use of personal protective equipment during mastoidectomy for COVID-19 patients.

Acknowledgments

We would like to thank Editage (www.editage.com) for English language editing.

References

1. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020;395:497-506.

2. Huang X, Wei F, Hu L, Wen L, Chen K. Epidemiology and Clinical Characteristics of COVID-19. *Arch Iran Med* 2020;23:268-271

3. Lechien JR, Chiesa-Estomba CM, De Siati DR, et al. Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. *Eur Arch Otorhinolaryngol* 2020;1-11.

4. Vaira LA, Deiana G, Fois AG, et al. Objective evaluation of anosmia and ageusia in

189 COVID-19 patients: Single-center experience on 72 cases [published online ahead of
190 print, 2020 Apr 27]. *Head Neck* 2020;10.1002/hed.26204.

191 5. Pitkäranta A, Virolainen A, Jero J, et al. Detection of rhinovirus, respiratory syncytial
192 virus, and coronavirus infections in acute otitis media by reverse transcriptase
193 polymerase chain reaction. *Pediatrics* 1998;102:291-295.

194 6. Bulut Y, Güven M, Otlu B, et al. Acute otitis media and respiratory viruses. *Eur J*
195 *Pediatr* 2007;166:223-228.

196 7. Pitkäranta A, Jero J, Arruda E, et al. Polymerase chain reaction-based detection of
197 rhinovirus, respiratory syncytial virus, and coronavirus in otitis media with effusion. *J*
198 *Pediatr* 1998;133:390-394.

199 8. Fidan V. New type of corona virus induced acute otitis media in adult [published
200 online ahead of print, 2020 Apr 16]. *Am J Otolaryngol* 2020;102487.

201 9. Sriwijitalai W, Wiwanitkit V. Hearing loss and COVID-19: A note [published online
202 ahead of print, 2020 Apr 2]. *Am J Otolaryngol* 2020;102473.

203 10. Mustafa MWM. Audiological profile of asymptomatic Covid-19 PCR-positive cases
204 [published online ahead of print, 2020 Apr 10]. *Am J Otolaryngol* 2020;102483.

205 11. Li W, Moore MJ, Vasilieva N, et al. Angiotensin-converting enzyme 2 is a
206 functional receptor for the SARS coronavirus. *Nature* 2003;426:450-454.
207 12. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory
208 disease in China [published correction appears in Nature. 2020 Apr;580(7803):E7].
209 *Nature* 2020;579:265-269.
210 13. Lukassen S, Chua RL, Trefzer T, et al. SARS-CoV-2 receptor ACE2 and TMPRSS2
211 are primarily expressed in bronchial transient secretory cells. *EMBO J*
212 2020;39:e105114.
213 14. Walls AC, Park YJ, Tortorici MA, et al. Structure, Function, and Antigenicity of the
214 SARS-CoV-2 Spike Glycoprotein. *Cell* 2020;181:281-292.e6.
215 15. Coutard B, Valle C, de Lamballerie X, et al. The spike glycoprotein of the new
216 coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same
217 clade. *Antiviral Res* 2020;176:104742.
218 16. Matsuyama S, Nao N, Shirato K, et al. Enhanced isolation of SARS-CoV-2 by
219 TMPRSS2-expressing cells. *Proc Natl Acad Sci U S A* 2020;117:7001-7003.
220 17. Guipponi M, Toh MY, Tan J, et al. An integrated genetic and functional analysis of

221 the role of type II transmembrane serine proteases (TMPRSSs) in hearing loss. *Hum*
222 *Mutat* 2008;29:130-141.

223 18. R. Ueha, K. Kondo, R. Kagoya et al. Background mechanisms of olfactory
224 dysfunction in COVID-19: expression of ACE2, TMPRSS2, and Furin in the nose and
225 olfactory bulb in human and mice. submitted
226 https://biorxiv.org/cgi/content/short/2020.05.15.097352v1

227 19. Chen JX, Workman AD, Chari DA, et al. Demonstration and mitigation of aerosol
228 and particle dispersion during mastoidectomy relevant to the COVID-19 era [published
229 online ahead of print, 2020 May 8]. *Otol Neurotol.*
230 2020;10.1097/MAO.0000000000002765.

231 20. Sharma D, Rubel KE, Ye MJ, et al. Cadaveric Simulation of Otologic Procedures:
232 An Analysis of Droplet Splatter Patterns During the COVID-19 Pandemic [published
233 online ahead of print, 2020 May 19]. *Otolaryngol Head Neck Surg*
234 2020;194599820930245.

235 21. Almufarrij I, Uus K, Munro KJ. et al. Does coronavirus affect the audio-vestibular
236 system? A rapid systematic review. *Int J Audiol.* 2020;12:1-5

237

Figure Legends

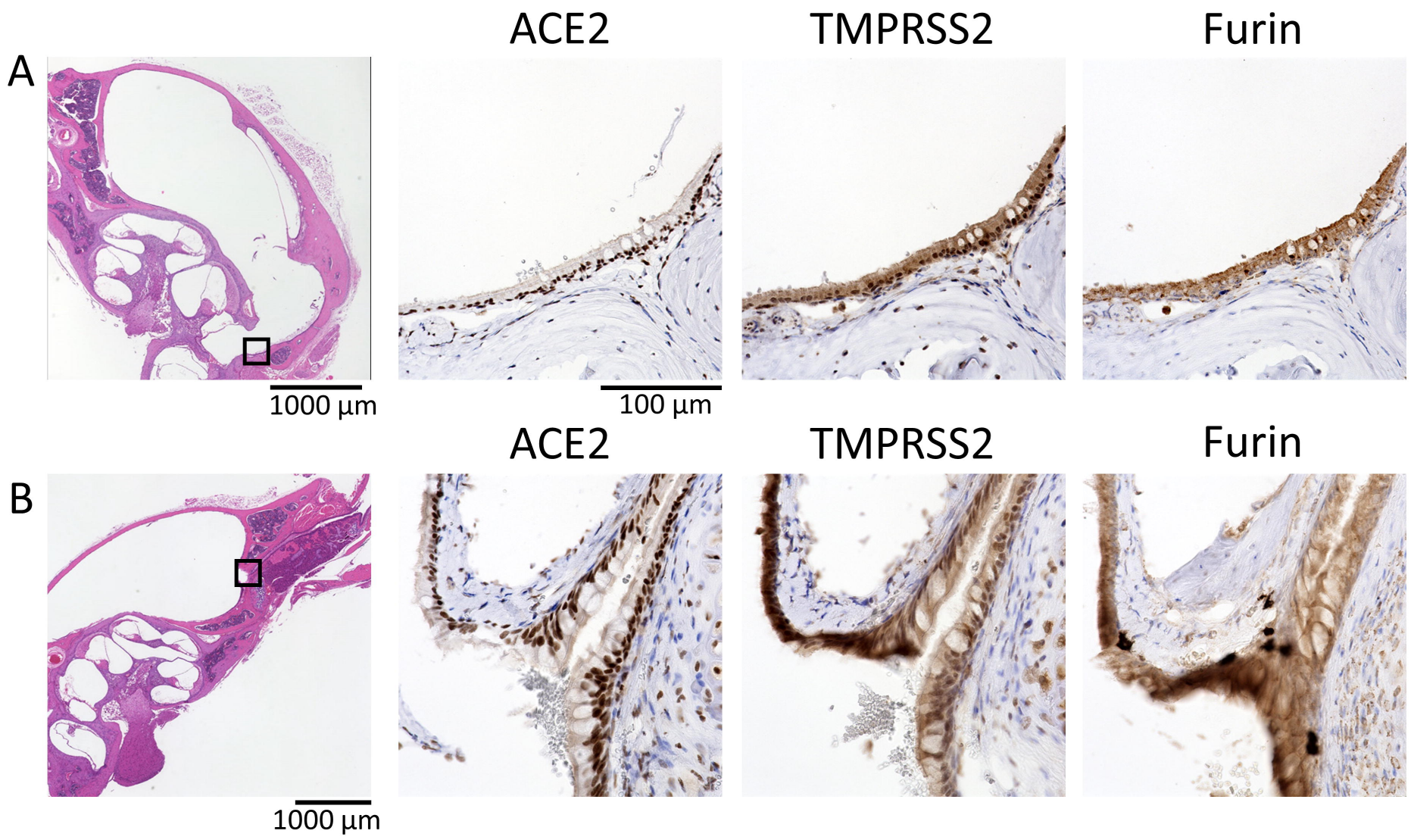
Figure 1. Histological analysis of the middle ear spaces and the Eustachian tube expressing Ace2, Tmprss2, and Furin.

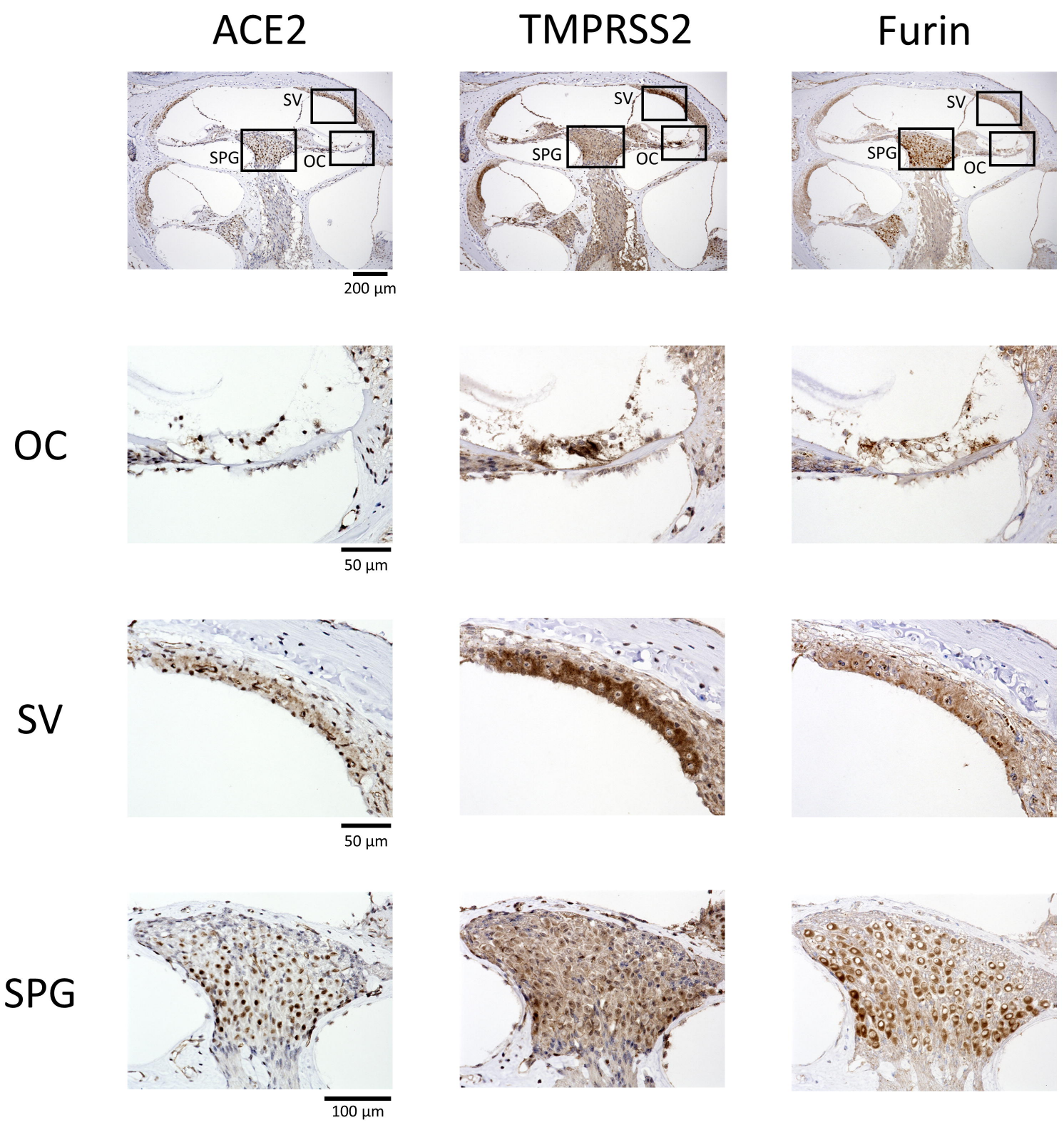
A: Hematoxylin-eosin staining of the middle ear mucosa (magnification, 40×). Immunohistochemical staining for Ace2 (cytoplasm), Tmprss2 (nucleus and cytoplasm), and Furin (cytoplasm) (400×). **B:** Parts of the Eustachian tube (40×) and Ace2, Tmprss2, and Furin expression in middle ear spaces (400×).

Figure 2: Cochlear Ace2, Tmprss2, and Furin expression.

Top panel: Parts of the organ of Corti (OC), stria vascularis (SV), and spiral ganglion (SPG) (magnification, 40×). **OC**: Ace2 expressed in the nucleus of the hair cells and OC supporting cells; TMPRSS2, diffuse in OC cell nuclei and cytoplasm; Furin, diffuse in the OC cell cytoplasm. **SV**: Ace2, nucleus of marginal, intermediate, and basal cells in the stria vascularis; Tmprss2, nucleus and cytoplasm in the stria vascularis; Furin, cytoplasm in the stria vascularis. **SPG**: Ace2, nucleus of the spiral ganglion cells; Tmprss2, diffuse in the nucleus and cytoplasm in spiral ganglion cells; Furin, diffuse in

254 the cytoplasm in the spiral ganglion cells.


ACE2
TMPRSS2
Furin
A
1000 µm
100 µm
ACE2
TMPRSS2
Furin
B
1000 µm


ACE2
TMPRSS2
Furin
SV
SPG
OC
200 µm
OC
50 µm
SV
50 µm
SPG
100 µm