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Background mechanisms of olfactory dysfunction in COVID-19: expression of ACE2, TMPRSS2, and Furin in the nose and olfactory bulb in human and mice

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ACE2, TMPRSS2, and Furin expression in the nose and olfactory bulb in mice and humans*

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To the Editor:

The coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) results in numerous clinical symptoms, including a deteriorated sense of taste and smell and respiratory and digestive disorders⁽¹⁾. Anosmia, the loss of smell, is a common clinical feature observed in the early stages of COVID-19. SARS-CoV-2 entry into the host cell depends on several factors, including the binding of viral spike proteins to the cellular receptor angiotensin-converting enzyme 2 (ACE2)^(2,3), spike protein cleavage by the host cell enzyme, Furin^(4,5), and spike protein priming by host cell proteases such as transmembrane protease serine 2 (TMPRSS2)⁽²⁾. Thus, high expression of ACE2, TMPRSS2, and Furin is thought to enhance SARS-CoV-2 entry, as well as clinical symptoms. To elucidate the mechanisms underlying olfactory dysfunction in COVID-19 patients, we investigated the expression of ACE2, TMPRSS2, and Furin in the respiratory mucosa (RM), olfactory mucosa (OM), and olfactory bulb (OB) of mouse and human tissues using immunohistochemistry and gene analyses.

A detailed explanation of the methods is provided in Supplemental Note 1. Briefly, animal tissue samples (6 eight-week-old male C57BL/6 mice; an eight-week-old male Sprague Dawley rat) examined in a previously published study (6, 7) and human tissues (OM, n = 3; middle turbinate, n = 5; inferior turbinate, n = 6) obtained from patients undergoing surgery for chronic rhinosinusitis (6 patients) and neuroblastoma (3 patients) were used for immunohistochemistry, and the expressions of ACE2, TMPRSS2, and Furin in the RM, OM, and OB were evaluated. In addition, our previous DNA microarray data (6) from the nasal mucosa, including RM and OM and OB (GSE 103191, 150694) were used to examine the expression of *Ace2*, *Tmprss2*, and *Furin* in mice.

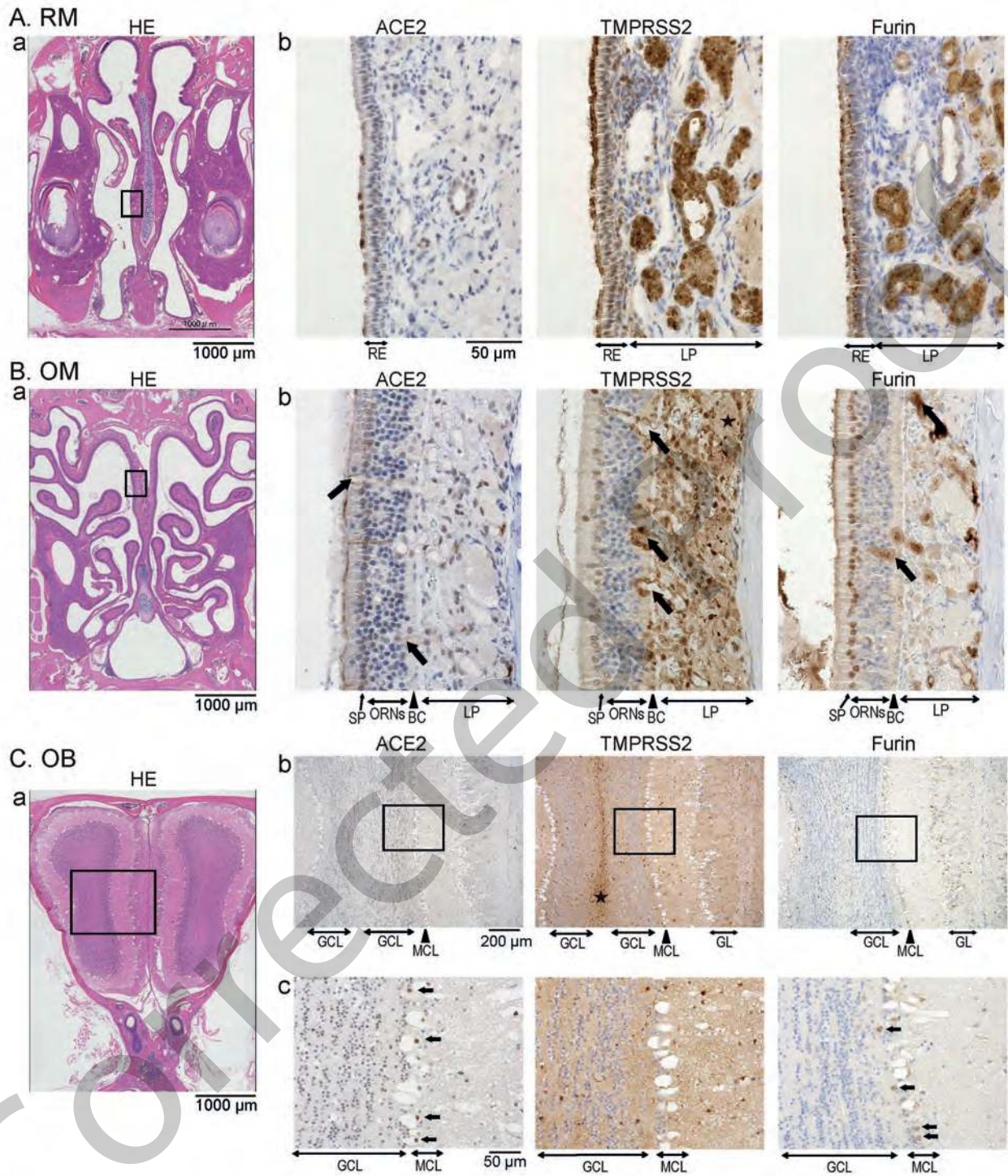
The immunohistological data are summarized in Table 1. In

Table 1. Protein expression levels of ACE2, TMPRSS2, and Furin in each tissue of mice and humans.

		ACE2	TMPRSS2	Furin
RM	RE Epithelial cell	±	+(m), ±(h)	-
	Cytoplasm of RE cell	±	+	+
LP	Subepithelial Gland	-	++	++
	Microvilli of supporting cell	±	+	+
OE	Cytoplasm of supporting cell	+(m), -(h)	+	+
	Olfactory receptor neuron	±(m), -(h)	-	-
OM	Basal cell	-	±	-
	Bowman's Gland	+(m), -(h)	++	+
	Olfactory nerve bundle	-	+	-
OB	Glomerular layer (m)	+	-	+
	Mitral cell layer (m)	+	-	+
	Granule cell layer (m)	+	-	-

ACE2: angiotensin-converting enzyme 2; TMPRSS2: transmembrane protease serine 2; RM: respiratory mucosa; RE: respiratory epithelium; LP: lamina propria; OM: olfactory mucosa; OE: olfactory epithelium; OB: olfactory bulb; ±: sparse expression; +: mild expression; ++: strong expression; -: negative; (m): mouse tissue; (h): human tissue.

mouse RM, ACE2, TMPRSS2, and Furin were all expressed in the cilia and cytoplasm of respiratory epithelial cells (Figure 1A-a). TMPRSS2 and Furin were highly co-expressed in the respiratory epithelium (RE). In OM, only supporting cells and Bowman's glands expressed ACE2, TMPRSS2, and Furin. Notably, olfactory receptor neurons (ORNs) and basal cells, except for supporting cells, were sparsely positive for ACE2, and the ORNs were negative for TMPRSS2 and Furin (Figure 1B). In the OB, there were



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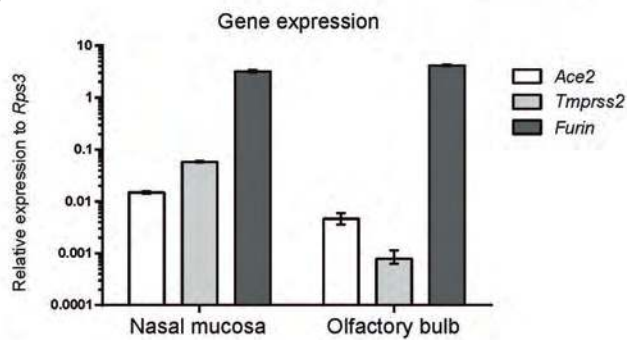


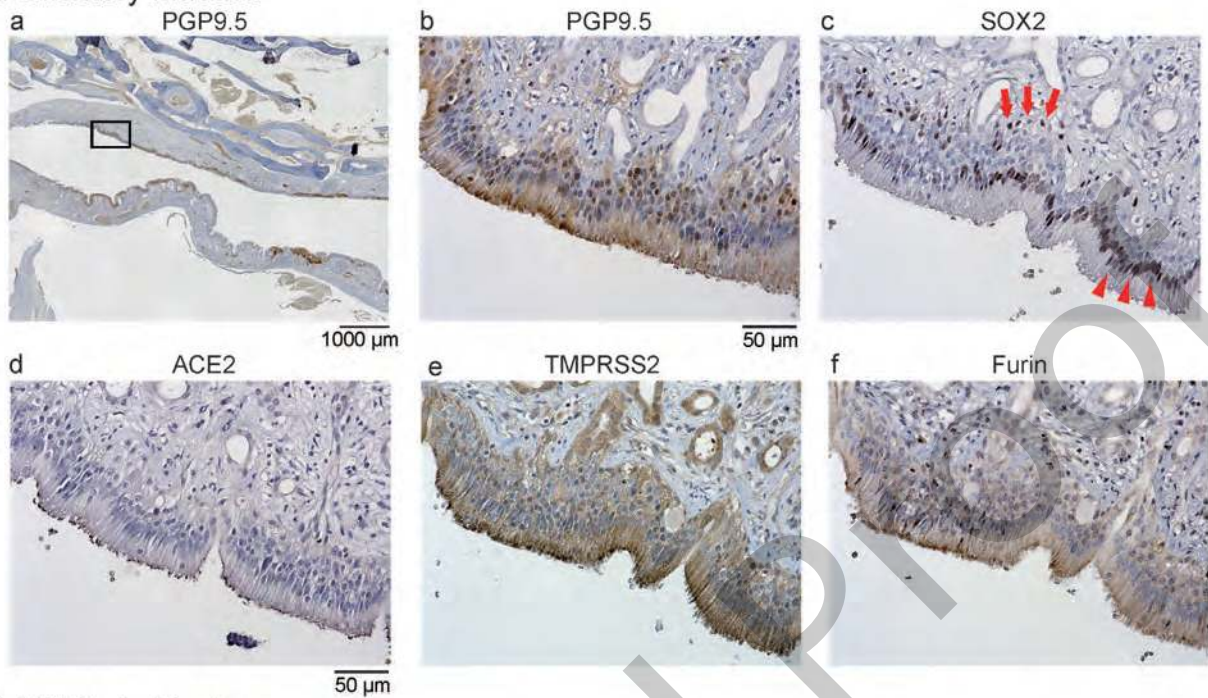
Figure 1. Representative images of mouse respiratory and olfactory mucosa and olfactory bulb stained with antibodies against angiotensin-converting enzyme 2 (ACE2), transmembrane protease serine 2 (TMPRSS2), and Furin. A: The box in (a) (the hematoxylin and eosin (HE) stained image) indicates the parts of the respiratory mucosa (RM) that is shown at higher magnification in (b) (a, 40x magnification; b, 400x magnification). In the RM area, the respiratory epithelium (RE) was hardly stained with an ACE2 antibody, except for the cilia of the ciliated columnar cells. In contrast, TMPRSS2 and Furin were strongly stained in the cilia of the ciliated columnar RE and moderately stained in the supranuclear cytoplasm of RE cells and sub-epithelial glands. B: The box in the HE stained image indicates the part of the olfactory mucosa (OM) that is shown at higher magnification in other images (a, 40x magnification; b, 400x magnification). In the OM area, supporting cells (SPs, arrows) and Bowman's glands (thick black arrows) were mildly stained with the ACE2 antibody, whereas olfactory receptor neurons (ORNs) and basal cells (BCs, arrowheads) were hardly stained with ACE2. TMPRSS2 expression was negative in ORNs, moderately positive in SPs, and strongly positive in Bowman's glands (thick black arrows) and the lamina propria. The olfactory nerve bundles showed weak expression of TMPRSS2 (star marks). Furin was also expressed in SPs and Bowman's glands (thick black arrows). C: The olfactory bulb (OB) area. The boxes in (a) and (b) are shown at higher magnification in (b) and (c), respectively (a, 40x magnification; b, 100x magnification; c, 400x magnification). The OB parenchyma was positive for TMPRSS2. While ACE2 positive cells could be sparsely recognized in the glomerular layer (GL) and granule cell layer (GCL), those cells were negative for TMPRSS2 in the mitral cell layer explicitly (MCL, arrowheads). Some mitral cells were positive for Furin (thick black arrows). TMPRSS2 was strongly expressed in the cells of the OB core (star marks). D: Gene expression levels of *Ace2*, *Tmprss2*, and *Furin* in the nasal mucosa and olfactory bulb are shown relative to the expression of the endogenous control gene *Rps3* (encoding ribosomal protein S3). The graph is shown in log10 scale.

no cells expressing ACE2, TMPRSS2, and Furin simultaneously (Figure 1C). ACE2-positive cells were sparsely detected in the glomerular layer, mitral cell layer, and granule cell layer; however, those cells were negative for TMPRSS2. In contrast, some cells in the glomerular layer and the mitral cells were positive for Furin. To reinforce these histological results, we used DNA microarray data from a previous study⁽⁶⁾, and then confirmed *Ace2*, *Tmprss2*, and *Furin* expressions in mouse nasal mucosa and OB (Figure 1D). In human nasal mucosa, the PGP9.5 antibody clearly stained the OE-containing olfactory neurons (Figure 2A-a,b). The supporting cells and the basal cells of the OE were recognized by SOX2 staining (Figure 2A-c). ACE2 was not present in the OE except for the cilia or microvilli, whereas TMPRSS2 and Furin were expressed in the apical layer of the human OE (Figure 2A-d,e,f). In the RM, TMPRSS2 and Furin were widely co-expressed in the epithelium, but ACE2 was expressed only in the respiratory cilia (Figure 2B). These findings were basically similar to those of the mouse tissues, although the ACE2 expression patterns in the supporting cells and Bowman's glands are different between mice and humans.

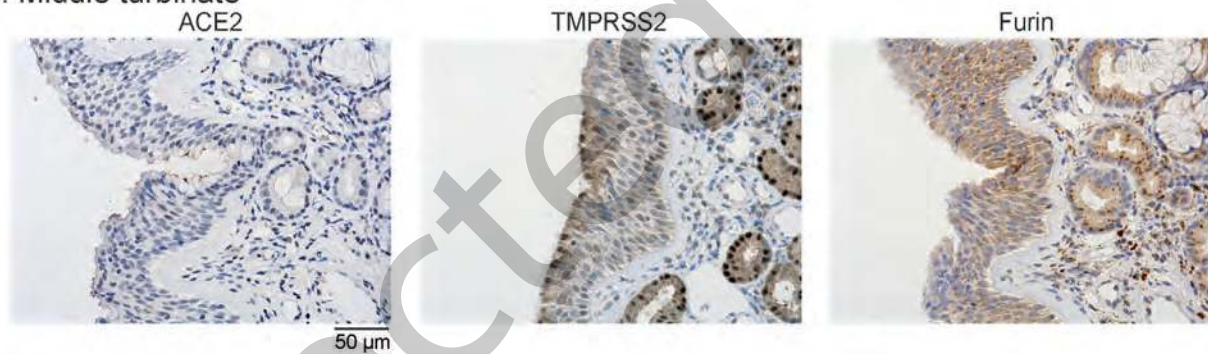
The results of this study can partially explain why olfaction is frequently impaired in COVID-19 patients. Although there have been several reports regarding the expression of ACE2 and TMPRSS2 in the nasal mucosa and olfactory bulb^(3,8), this was the first report regarding the expression of Furin in these tissues. Olfactory dysfunction is classified into three types according to the anatomical location, specifically, conductive, sensorineural, and central. Conductive dysfunction results from the blockage of odorant airflow to the OE. Sensorineural dysfunction is caused by damage to the ORNs and the olfactory nerve. Central dysfunction occurs with the damage of olfactory processing pathways in the central nervous system.

Considering the high expression of ACE2, TMPRSS2, and Furin in the RE and subepithelial glands, SARS-CoV-2 possibly induces conductive olfactory dysfunction through hypersecretion and goblet cell hyperplasia. In fact, a significant number of COVID-19 patients suffer from nasal obstruction and rhinorrhea⁽¹⁾. However, considering that a certain proportion of COVID-19 patients with hyposmia and anosmia did not exhibit nasal obstruction or rhinitis symptoms⁽¹⁾ and the RE has less ACE2 expression than the OE⁽⁸⁾, the contribution of conductive olfactory loss might be limited. ACE2, TMPRSS2, and Furin were co-expressed in the supporting cells and Bowman's glands of the OM, as well as in the RM, but not in the ORNs or the OB, which suggest that SARS-CoV-2 might mainly induce sensorineural olfactory dysfunction with less olfactory neuronal damage, as well as induce the deterioration of mucus production and OE support. It is unlikely that SARS-CoV-2 directly damages the ORNs in the OM, because the ORNs expressed neither ACE2 nor TMPRSS2/Furin. Other hypotheses behind COVID-19 related smell loss are considered, such as inflammatory processes in the OE, loss of support for ORNs, change in composition, or/and ionic concentration in the olfactory mucus, the olfactory system as a door to central infection, and alteration of olfactory bulb function. The co-expression of ACE2 and Furin in the mitral cells of the OB, which have large cell bodies and secondary dendrites, suggests that central olfactory dysfunction might occur as a result of synaptic inhibition from the ORNs to the olfactory bulb. Although most COVID-19 patients recover from olfactory dysfunction, some patients have not recovered their olfaction after several months⁽⁹⁾. Persistent olfactory dysfunction could be due to sensorineural loss that is known to recover slowly (e.g., postinfectious olfactory loss) or central olfactory dysfunction. Future studies are needed for clinical evaluation with long-term-follow-up. Regarding the limitations of this study, the evaluations were

A. Olfactory mucosa



B. Middle turbinate



C. Inferior turbinate

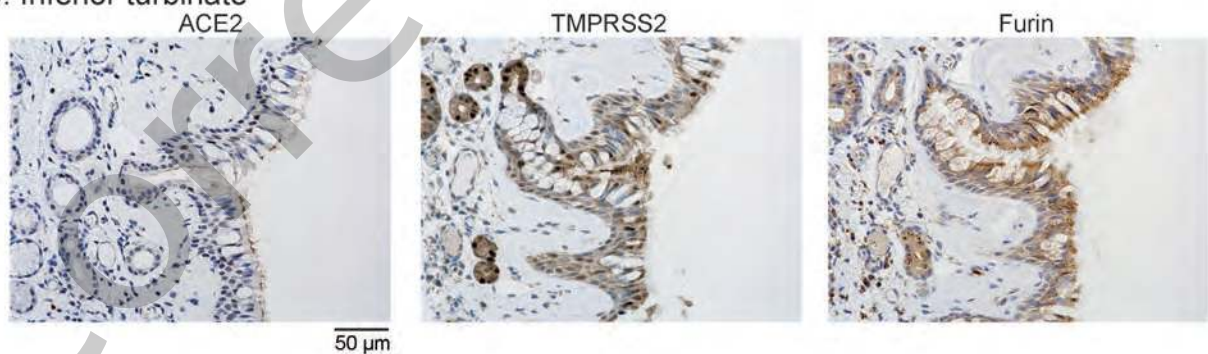


Figure 2. Representative images of human tissues stained with antibodies against angiotensin-converting enzyme 2 (ACE2), transmembrane protease serine 2 (TMPRSS2), and Furin. A: (a) In the olfactory mucosal area, the area of the olfactory epithelium (OE) was recognized using PGP9.5 staining. The box in (a) indicates the part of the olfactory mucosa that is shown at higher magnification in (b-f) (a, 40x magnification; b-f, 400x magnification). (c) SOX2 was expressed in the basal cells (red arrows) and the supporting cells (red arrowheads) of the OE. (d) ACE2 was noted in the microvilli or cilia of the OE cells, but not in the olfactory receptor neurons (ORNs). (e, f) Human ORNs expressed neither TMPRSS2 nor Furin. There was a high expression of TMPRSS2 in the cytoplasm of the subepithelial glands. Furin was rarely detected in the human olfactory epithelium, but a weak expression was noted in the subepithelial glands. B and C: Representative images of the middle turbinate and inferior turbinate (400x magnification). ACE2 expression was clearly recognized in the respiratory cilia, but mild in the supranuclear cytoplasm of RE cells. TMPRSS2 and Furin expression was detected in the respiratory epithelium and subepithelial glands. Specifically, TMPRSS2 expression was well detected.

performed on mice and some patients with olfactory neuroblastoma, and thus, may not be representative of functioning olfactory mucosa nor correctly reflect the background of SARS-CoV-2 infection despite the use of areas without tumor lesions. Future clinical case and autopsy studies might strengthen the results of this study.

Conflict of interest

There are no competing interests.

Authorship contribution

RU developed the concept, designed and performed the

experiments, and wrote the draft of the manuscript. RK and SS designed and performed the experiments. SU performed the experiments and partly wrote the draft of the manuscript. KK and TY developed the concept, designed the experiments, and reviewed the manuscript. All authors contributed to the interpretation of the data and writing of the manuscript.

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SUPPLEMENTARY DATA

Materials and Methods

Experimental samples

Animal tissue samples were all obtained from the mice examined in previously published studies (1, 2) because purchasing new animals is prohibited in our facility due to the epidemic spread. Samples from 6 eight-week-old male C57BL/6 mice (2) and an eight-week-old male Sprague Dawley rat (3) were used, and the following paraffin-embedded tissues were collected; the respiratory mucosal (RM) area and olfactory mucosal (OM) area of the nose, the olfactory bulb (OB) area, and the kidney and prostate for positive controls of immunostaining (Supplemental figure 1). Human tissues were obtained from patients undergoing surgery for the treatment of chronic sinusitis (6 patients) or olfactory neuroblastoma (3 patients). These included the OM (n = 3), the middle turbinate (n = 5), and inferior turbinate (n = 6); the latter two were used for the evaluation of the RM. Routine morphology was evaluated in hematoxylin and eosin-stained sections by qualified pathologists and otolaryngologists. Tissue evaluation was performed only in the parts characterized as non-diseased. All experiments were conducted in accordance with institutional guidelines and with the approval of the Animal Care and Use Committee of the University of Tokyo (No. P14-051, P15-115) and of the Research Ethics Committee of the Graduate School of Medicine and Faculty of Medicine, the University of Tokyo, Japan (12009, 2019073NI). Since archived specimens were used, written informed consent was waived.

Histological analyses

To detect the expressions of ACE2 and TMPRSS2 in the RM, OM, and OB, histological analyses were performed by immunostaining. Four micrometers-thick serial paraffin sections were deparaffinized in xylene and dehydrated in ethanol before immunostaining. Before immunostaining, deparaffinized sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity and were incubated in Blocking One (Nacal Tesque, Kyoto, Japan) to block non-specific immunoglobulin

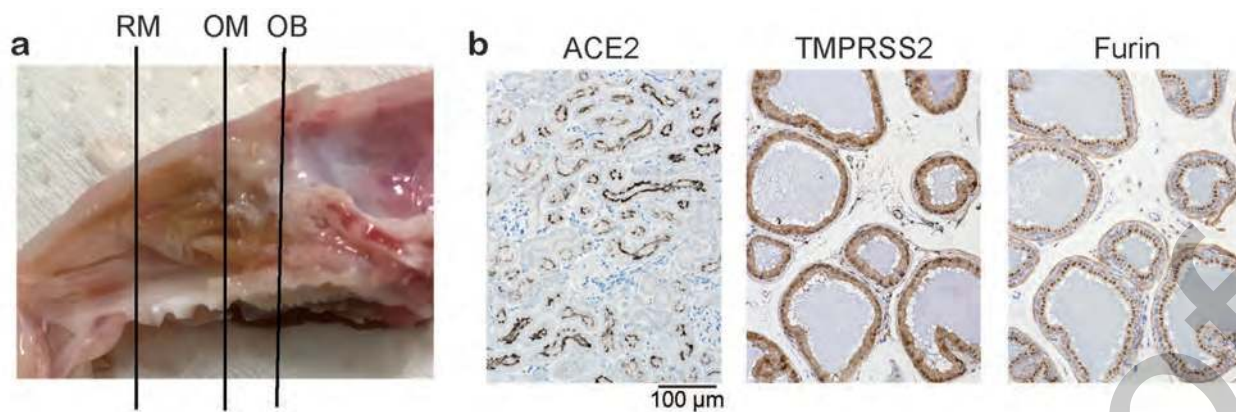
binding. After antigen activation, primary antibodies against ACE2 (1:300 dilution; rabbit monoclonal, Abcam, ab108252; Cambridge, UK), TMPRSS2 (1:1000 dilution; rabbit monoclonal, Abcam, ab92323; Cambridge, UK), Furin (1:100 dilution; rabbit monoclonal, Abcam, ab183495; Cambridge, UK), PGP9.5 for a neuronal marker (1:500 dilution; guinea pig polyclonal, Abcam, ab10410; Cambridge, UK), and SOX2 for supporting cells and horizontal basal cells in the olfactory epithelium (1,2) (1:300 dilution; rabbit monoclonal, Abcam clone EPR3131; Cambridge, UK) were detected with appropriate peroxidase-conjugated secondary antibodies and a diaminobenzidine substrate. The mouse kidney and rat prostate were stained as positive controls for ACE2 and the TMPRSS2 and Furin, respectively (Figure 1B). All samples were stained under the same condition and protocol as those of the positive control staining. Images of all sections were captured using a digital microscope camera (Keyence BZ-X700) with 4x, 10x, 20x, and 40x objective lenses.

Gene expression analyses

Our previous DNA microarray data (1, 2) from the nasal mucosa and OB (NCBI Gene Expression Omnibus database under the series number GSE 103191, 150694) were used to examine the expressions of ACE2, TMPRSS2, and Furin. The expression levels of each gene were normalized against the expression level of Rps3 (encoding ribosomal protein S3) in each sample.

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Supplementary Figure 1. Nose sagittal section and representative images of angiotensin-converting enzyme 2 (ACE2) staining in mouse kidney and transmembrane protease serine 2 (TMPRSS2) and Furin staining in rat prostate. A: Sagittal section of the rodent nose. The bars indicate the respiratory mucosal section (RM), olfactory mucosal section (OM), and the olfactory bulb section (OB). B: Positive staining for ACE2 in the kidney and TMPRSS2 and Furin in the prostate are shown. ACE2-positive cells are observed in the brush border of tubular cells. TMPRSS2-positive cells are observed in the cytoplasm and nucleus of acinar cells. Furin-positive cells are observed in the supranuclear cytoplasm (mainly Golgi apparatus) of acinar cells in the prostate glands.