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**Expression of indian hedgehog signaling in murine oviductal infundibulum and its
relationship with epithelial homeostasis**

Authors: Marina Hosotani^{1*}, Osamu Ichii^{2,3}, Takashi Namba², Md. Abdul Masum², Teppei

Nakamura^{2,4}, Yasuhiro Hasegawa⁵, Takafumi Watanabe¹, Yasuhiro Kon²

Addresses: ¹Laboratory of Veterinary Anatomy, School of Veterinary Medicine, Rakuno Gakuen

University, Ebetsu, Hokkaido 069-8501, Japan

²Laboratory of Anatomy, Department of Basic Veterinary Science, Faculty of Veterinary

Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

³Laboratory of Agrobiomedical Science, Faculty of Agriculture, Hokkaido University, Sapporo,

Hokkaido 060-0818, Japan

⁴Department of Biological Safety Research, Chitose Laboratory, Japan Food Research

Laboratories, Chitose, Hokkaido 066-0052, Japan

⁵Department of Food Science and Human Wellness, College of Agriculture, Food and

Environment Science, Rakuno Gakuen University, Ebetsu, Japan

***Corresponding author: Marina Hosotani, DVM,**

Laboratory of Veterinary Anatomy, School of Veterinary Medicine, Rakuno Gakuen University,

Midorimachi 582, Bunkyo-dai, Ebetsu 069-8501, Japan

19 Tel & Fax: +81-11-388-4763

20 Email: m-hosotani@rakuno.ac.jp

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Abstract

Homeostasis of the oviductal infundibulum epithelium is continuously regulated by signaling pathways under physiological and pathological conditions. Herein, we investigated the expression of hedgehog (Hh) signaling-related components in the murine oviductal infundibulum, which is known to maintain homeostasis in the adult epithelium. Additionally, using autoimmune disease-prone MRL/MpJ-Fas^{lpr/lpr} (MRL/lpr) mice showing abnormal morphofunction of the ciliated epithelium of the infundibulum related to the oviductal inflammation, we examined the relationship between Hh signaling and pathology of the infundibulum. The expression and localization of Pax8, a marker for progenitor cells in the oviductal epithelium, and Foxj1, a marker for ciliogenesis, were examined in the infundibulum. The results showed that Pax8 was downregulated and Foxj1 was upregulated with aging, suggesting that homeostasis of the infundibulum epithelium of MRL/lpr mice was disturbed at 6 months of age. In all mice, the motile cilia of ciliated epithelial cells in the infundibulum harbored Hh signaling pathway-related molecules: patched (Ptch), smoothened (Smo), and epithelial cells harbor Gli. In contrast, *Ptch*, *Smo*, and *Gli2* were significantly downregulated in the infundibulum of MRL/lpr mice at 6 months of age. The expression levels of *Pax8* and *Foxj1* were significantly positively correlated with those of *Ptch1*, *Smo*, and *Gli2*. Hh signaling is thought to be involved in homeostasis of the ciliated epithelium in the infundibulum. In MRL/lpr mice, which show exacerbated severe systemic

46 autoimmune abnormalities, molecular alterations in Hh signaling-related components are
47 considered to interact with local inflammation in the infundibulum, leading to disturbances in
48 epithelial homeostasis and reproductive function.

49

50 **Keywords:** autoimmune abnormality, ciliated epithelium, hedgehog signaling pathway,
51 homeostasis, oviduct

Introduction

Mammalian oviducts are divided into three parts, the infundibulum, ampulla, and isthmus, in order from the distal to proximal parts. The different composition ratios of ciliated epithelial cells and secretory cells in the epithelium of each part reflect the unique reproductive function in each section (Li et al. 2017; Koyama et al. 2019). Particularly, the infundibulum epithelium is composed mostly of ciliated epithelial cells, so that ciliary beating effectively moves oocytes produced in the ovaries into the oviductal lumen; this process is known as oocyte pick-up. Oocyte pick-up and transportation are disturbed by abnormal ciliary morphofunction in the infundibulum caused by pathological conditions such as smoking (Talbot and Riveles 2005), hormonal dysregulation (Raidt et al. 2015), and inflammation (Hosotani et al. 2020). Physiologically, the proportion of ciliated epithelial cells and secretory cells in the infundibulum epithelium changes under hormonal dynamics through the estrous cycle (Ito et al. 2016). In addition, the infundibulum is constantly exposed to follicular fluid containing inflammatory molecules during each ovulation, which damages the ciliary epithelium (Palma-Vera et al. 2017). Therefore, the infundibulum epithelium continuously undergoes epithelial turnover to maintain its healthy histology and reproductive function under both physiological and pathological conditions.

Adult epithelial homeostasis is maintained by the proliferation and differentiation of epithelial stem cells, which are regulated by activation of signaling pathways such as the Wnt/ β -catenin,

Notch, and Hedgehog (Hh) signaling pathways (Sancho et al. 2004; Carlier et al. 2020). In oviductal epithelial homeostasis, secretory cells act as progenitors by self-renewing and/or differentiating into ciliated epithelial cells (Ghosh et al. 2017). The molecular mechanism underlying these effects is not fully understood; however, several studies have focused on the involvement of Wnt/ β -catenin signaling (Ghosh et al. 2017) and Notch signaling (Zhu et al. 2019) in homeostasis of the oviductal epithelium. In contrast, although homeostasis of the adult tracheal ciliated epithelium is maintained by activation of the Hh signaling pathway (Peng et al. 2015), the involvement of this pathway in maintaining the oviductal epithelium has not been explored.

In a murine model of systemic autoimmune disease, MRL/MpJ-Fas^{lpr/lpr} (MRL/lpr) mice develop severe inflammation of the lamina propria in the oviductal infundibulum. Chronic abnormal immune conditions result in abnormal morphofunction in the ciliated epithelium, such as decreased numbers of ciliated epithelial cells, elongation of the cilia, and disorientation of ciliary beating (Hosotani et al. 2018, 2020). These pathological conditions are closely related to disturbances in epithelial homeostasis but the underlying molecular mechanism is unclear.

In this study, we investigated expression of the Hh signaling pathway in the murine infundibulum epithelium and its relationship with homeostasis of the infundibulum epithelium. In addition, we examined the expression of Hh signaling pathway-related molecules in MRL/lpr mice as a destruction model of the infundibulum epithelium and compared the results with those

- 88 obtained the healthy infundibulum of C57BL/6N mice (B6) as a general strain and MRL/MpJ mice
- 89 (MRL/+) as wild-type MRL/lpr mice.

Material and methods

Animals

Animal experiments were approved by the School of Veterinary Medicine, Rakuno Gakuen University (approval no. VH19A6). The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, Rakuno Gakuen University, Japan. Female B6, MRL/+, and MRL/lpr mice at 3 and 6 months of age were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Previous studies reported that autoimmune disease is severely exacerbated in female MRL/lpr mice at 6 months of age compared to at 3 months of age (Hosotani et al. 2018, 2020). The mice were housed in groups within plastic cages at 18–26°C under a 12-h light/dark cycle and had free access to a commercial diet and water. The estrous cycle of each mouse under the natural estrous cycle was confirmed by monitoring vaginal smears (Byers et al. 2012). All mice were euthanized by either severing the carotid artery or cervical dislocation under deep anesthesia using a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). The spleen was collected from the mice and weighed as a marker of autoimmune disease.

Immunostaining

Mouse female reproductive organs were collected and fixed with 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and cut into 3-μm-thick sections, which were then used for

immunohistochemistry (IHC) and immunofluorescence (IF). Detailed information on the antibodies, antigen retrieval, and serum blocking is listed in Table 1. The sections were incubated in 20 mM Tris-HCl (pH 9.0) for 15 min at 110°C, 10 mM citrate buffer (pH 6.0) for 15 min at 110°C, or 0.1% pepsin for 5 min at 37°C. The sections for IHC were soaked in methanol containing 0.3% hydrogen peroxide. Sections incubated with blocking serum for 60 min at room temperature were incubated overnight at 4°C with primary antibodies. Negative controls were performed with normal mouse IgG (sc-2025, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), normal rat IgG (sc-2026, Santa Cruz Biotechnology) and normal rabbit IgG (sc-2027, Santa Cruz Biotechnology). After three washes in 0.01 M PBS, the sections were incubated with secondary antibodies for 30 min and washed. The sections for IHC were incubated for 30 min at room temperature, using a streptavidin-biotin complex (SABPRO Kit, Nichirei, Tokyo, Japan), and then incubated with 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide solution, and lightly stained with hematoxylin. The stained sections of the outer infundibulum were examined using a BZ-X710 microscope (Keyence, Osaka, Japan).

Three semi-serial sections stained with IHC for detection of Foxj1 with 50-μm intervals were used, and the percentage of expelled non-ciliated epithelial cells and the percentage of Foxj1 positive expelled non-ciliated epithelial cells were calculated as follows, respectively: percentage of expelled non-ciliated epithelial cells (%) = $100 \times \text{number of expelled non-ciliated epithelial}$

cells / number of total 80-100 epithelial cells in the field. Percentage of Foxj1 positive expelled non-ciliated epithelial cells (%) = $100 \times \text{number of Foxj1 positive expelled non-ciliated epithelial cells} / \text{number of total 10-20 expelled non-ciliated epithelial cells in the field.}$

Reverse Transcription and Quantitative Real-time Polymerase Chain Reaction

The oviducts were manually separated into the proximal (including the isthmus and ampulla) and distal (including infundibulum) parts and homogenized using a BioMasher (Nippi Inc., Tokyo, Japan). Total RNA was purified using the NucleoSpin® RNA Plus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purified total RNA was used as a template to synthesize cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Quantitative real-time polymerase chain reaction (qPCR) analysis of the cDNA was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd.) and gene-specific primers (Table 2, Sigma-Aldrich, St. Louis, MO, USA). The qPCR cycling conditions were 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 52°C or 60°C for 45 s. Data were normalized against the expression level of actin, beta (*Actb*) and analyzed using the ΔCt method to compare the expression of genes encoding hedgehog ligands; the $\Delta\Delta\text{Ct}$ method was used to compare the expression of other genes.

In Situ Hybridization

Paraformaldehyde-fixed paraffin-embedded specimens of female reproductive organs were cut into 5µm-thick sections, air-dried overnight, and baked in an oven for 60 min at 60°C. RNA *in situ* hybridization was performed using an RNAscope® 2.5 HD Detection Regent-Brown kit (Advanced Cell Diagnostics, Newark, CA, USA) according to the manufacturer's instructions. The RNAscope® positive control probe-Mm-Polr2a (Cat. No. 312471, Advanced Cell Diagnostics), RNAscope® negative control probe-DapB (Cat. No. 310043, Advanced Cell Diagnostics), RNAscope® probe-Mm-Shh (Cat. No. 314361, Advanced Cell Diagnostics), RNAscope® probe-Mm-Dhh (Cat. No. 415031, Advanced Cell Diagnostics), and RNAscope® probe-Mm-Ihh-noXHs (Cat. No. 413091, Advanced Cell Diagnostics) were used.

Statistical Analysis

The results are expressed as the mean ± standard error (s.e.). Data among three or more groups were compared using Tukey's test ($P < 0.05$). Data between two groups were compared using Student's *t*-test ($P < 0.05$). Correlations between two parameters were analyzed using Spearman's correlation test ($P < 0.05$). Statistical analysis was conducted using JMP 14.2.0 (SAS Institute, Inc., Cary, NC, USA).

Result

Expression and Localization of Epithelial Homeostasis-Related Molecules in the Infundibulum

In the oviductal epithelium, Pax8 is a marker of secretory cells (i.e., progenitor cells), whereas Foxj1 is a marker of ciliogenesis. Pax8 was localized in the nucleus of secretory cells in the infundibulum of mice, except for in MRL/lpr mice at 6 months of age (Figure 1a-c and a'-c'). Foxj1 was localized in the nucleus of ciliated epithelial cells in the infundibulum of mice, except for MRL/lpr mice at 6 months of age, in which Foxj1 expression was observed not only in the nucleus of ciliated epithelial cells as well as that of non-ciliated epithelial cells expelled from the epithelium (Figure 1d-f and d'-f'). There was no significant differences in the percentage of expelled non-ciliated epithelial cells composing the infundibulum epithelium among the strains and ages (Figure 1h), while the percentage of Foxj1 positive expelled non-ciliated epithelial cells was significantly higher in MRL/lpr mice at 6 months of age than other strains and MRL/lpr mice at 3 months of age (Figure 1i).

We compared the transcriptional expression levels of *Pax8* and *Foxj1* in the infundibulum as the distal part of the oviduct with those in the ampulla and isthmus as the proximal part (Figure 2a and b). *Pax8* expression was lower in the distal part than in the proximal part of all strains at 3 months of age. In the entire oviduct of MRL/lpr mice at 6 months of age, *Pax8* expression showed an age-related decrease and was significantly lower than that in the other strains. In contrast, *Foxj1*

expression was higher in the distal part than in the proximal part of all mice. In the entire oviduct of MRL/lpr, *Foxj1* expression was higher than in other strains, particularly at the distal part which showed a significant age-related increase.

Expression and Localization of Hedgehog Signaling-Related Molecules in Infundibulum

The canonical Hh signaling pathway in primary cilia is activated by binding of Hh ligands such as sonic, indian, and desert Hh (Shh, Ihh, and Dhh, respectively) to the transmembrane protein patched (Ptch), followed by an interaction with smoothened (Smo) and the activation of GLI family zinc finger 1–3 (Gli1–3) transcription factors (Briscoe and Thérond 2013). There were no significant differences in the expression of Hh signaling pathway-related genes during the estrous cycle (Figure 4a-e); therefore, we focused on the infundibulum of mice at estrus. In all mice, Ptch1 and Smo were localized in the cilia of ciliated epithelial cells in the infundibulum (Figure 3a-a''', b-b''', c-c''', d-d''', e-e''', and f-f'''). The fluorescence intensity of Ptch1 and Smo in the infundibulum did not differ between mice of different strains and ages. In B6 and MRL/+ mice, Gli2 was highly expressed in the nucleus of secretory cells and faintly in the nucleus of ciliated epithelial cells in the infundibulum (Figure 3g-g''', h-h'''). In MRL/lpr mice at both 3 and 6 months of age, Gli2 was ubiquitously observed in the nuclei of cells comprising the infundibulum epithelium (Figure 3i-i''').

In transcriptional analysis, *Ptch1* expression did not significantly differ among the oviductal parts of all mice (Figure 5a). *Ptch1* expression showed an age-related decrease in the proximal part of B6 and at both the proximal and distal parts in MRL/lpr mice. In the entire oviduct of MRL/lpr mice, *Ptch1* expression was significantly lower than that in B6 or/and MRL/+ mice. *Smo* expression was significantly higher in the distal part than in the proximal part of B6 and MRL/+ mice at 3 months of age (Figure 5b). *Smo* expression showed an age-related decrease at the distal part in all mice. In the entire oviduct of MRL/lpr, *Smo* expression was significantly lower than that in the other strains. *Gli1* expression was significantly lower at the distal part than at the proximal part: *Gli2* and *Gli3* expression showed no significant differences among the oviductal parts (Figure 5c-e). In the entire oviduct of MRL/lpr, *Gli1* expression showed an age-related decrease and was significantly lower than that in the other strains (Figure 5c). The mean C_t value of *Gli1* expression in the distal parts was high above around 32 in all mice (data not shown), so that the localization level of *Gli1* protein seems to be very low in the distal part. In the entire oviduct of MRL/lpr, *Gli2* expression was significantly higher than in other strains, particularly at the distal part at 6 months of age (Figure 5d). *Gli3* expression tended to be higher in the entire oviduct of MRL/lpr mice at 3 months of age than in other strains, whereas that of MRL/lpr at 6 months of age showed an age-related decrease and was significantly lower than that in other strains (Figure 5e).

Among the transcription factors of the hedgehog ligands (*Shh*, *Dhh*, and *Ihh*), *Shh* and *Dhh*

were not expressed in the infundibulum epithelium or ovarian granulosa cells (Figure 6a-f and a'-f'), whereas *Ihh* was highly expressed in ovarian granulosa cells and slightly in infundibulum epithelial cells (Figure 6a''-f''). *Ihh* expression in ovarian granulosa cells was lower in MRL/lpr mice than in B6 mice (Figure 6a'' and e''). The *Shh* and *Dhh* expression levels were not significantly different among the oviductal parts, whereas *Ihh* expression was lower in the distal part than in the proximal part in all mice (Figure 7a-f).

Relation between Hh Signaling Pathway and Oviductal Epithelial Homeostasis

Correlation analysis of transcriptional expression in the oviduct of mice at 3 months of age was performed (Table 3). In the proximal part, *Ptch1* and *Pax8* expression was significantly positively correlated with the expression of all Hh signaling pathway-related genes. *Foxj1* expression showed the same results, except for *Gli1*. In contrast, at the distal part, *Ptch1* expression was significantly positively correlated with the expression of *Smo* and *Gli2* and significantly negatively correlated with *Gli3* expression. *Pax8* and *Foxj1* expression was significantly positively correlated with the expression of *Ptch1*, *Smo*, and *Gli2*.

Discussion

The molecular mechanism that maintains homeostasis of the infundibulum epithelium has been greatly altered in autoimmune disease model mice. This molecular alteration causes an abnormal morphology of the ciliated epithelium of the infundibulum of MRL/lpr mice at 6 months of age, such as a decreased proportion of ciliated epithelial cells, elongated cilia, and disorientation of ciliary alignment (Hosotani et al. 2020). The transcription factor Pax8 governs the expression of a series of genes pivotal to tissue development in thyroid follicular cells and Müllerian ducts (Plachov et al. 1990; Grote et al. 2006). Pax8 in the oviductal secretory cells is a marker of the self-renewal and differentiation of these cells to ciliated epithelial cells (Ghosh et al. 2017); therefore, the significant downregulation of Pax8 transcripts and proteins suggests that Pax8 attenuates epithelial homeostasis in the infundibulum of MRL/lpr at 6 months of age. The transcription factor Foxj1 is required for the late steps of ciliogenesis, including docking of centrioles at the apical membrane to form basal bodies and axoneme elongation (You et al. 2004). Foxj1 upregulation promotes abnormal ciliogenesis, resulting in disorganized, dense, and lengthened cilia in the airway ciliated cells of patients with chronic mucosal inflammation (Li et al. 2014). Considering that the direction of coordinated ciliary beating requires basal body polarization (Kunimoto et al. 2012), in the infundibulum of MRL/lpr mice at 6 months of age, significant upregulation of Foxj1 may promote abnormal ciliogenesis, resulting in cilia elongation

and disoriented ciliary alignment. Foxj1 localization in the expelled non-ciliated epithelial cells was significantly observed in the infundibulum of MRL/lpr mice at 6 months of age, which implies the increase of inadequate ciliogenesis and the promoted elimination of epithelial cells failed to differentiate to ciliated epithelial cells. Otherwise, significantly high expression of Foxj1 may have been a reaction to the decreased number of ciliated epithelial cells following disturbed ciliogenesis and loss of Pax8⁺ epithelial progenitor cells. The abnormal morphofunction of the ciliated epithelium of the infundibulum caused by alterations in homeostasis-related molecules causes dysfunction in oocyte pick-up (Hosotani et al. 2018, 2020).

This study revealed that the motile cilia of ciliated epithelial cells in the infundibulum harbor Hh signaling pathway-related molecules: the transmembrane receptors, Ptch and transmembrane protein adjacent to Ptch, and Smo (Carpenter et al. 1998). In addition, Hh signaling effectors in the Gli family are expressed in the nucleus of infundibulum epithelial cells. Considering the significant positive correlations between Ptch1 and Smo/Gli2 in the infundibulum, motile cilia in the infundibulum epithelium contain the Hh signaling pathway, although whether this pathway is canonical or non-canonical matter has not been determined. Although primary cilia (i.e., immotile cilia) are generally considered as sensor cilia in which canonical/non-canonical Hh signaling is transduced (Abou Alaiwi et al. 2009; Bangs and Anderson 2017), recent studies reported that motile cilia also act as sensors of the pericellular environment. The motile cilia of the tracheal

epithelium express sensing receptors such as the bitter taste receptor and *Ptch1*/*Smo* (Shah et al. 2009; Nordgren et al. 2014), which sense injury and chronic inflammation in the airway. The motile cilia in oviducts harbor progesterone receptors, which regulate the ciliary beat frequency (Teilmann et al. 2006; Bylander et al. 2010). Thus, motile cilia in the infundibulum epithelium may play a sensing role via Hh signaling-related components. Hh ligands were not significantly expressed in the infundibulum, and the present and previous studies (Russell et al. 2007) revealed *Ihh* expression in ovarian granulosa cells, where Hh signaling may induce granulosa cell proliferation. Therefore, cilia in the infundibulum may receive *Ihh* produced by ovarian granulosa cells at the time of ovulation, rather than through the paracrine effect of Hh produced by infundibulum epithelial cells.

In the infundibulum, *Pax8* and *Foxj1* expression showed a significant positive correlation with the gene expression of Hh signaling pathway-related *Ptch1*, *Smo*, and *Gli2*. This suggests that the Hh signaling pathway is closely related to the regulation of epithelial homeostasis in the infundibulum. To the best of our knowledge, direct molecular interactions between *Pax8* and/or *Foxj1* and the Hh signaling pathway during development and homeostasis maintenance have not been observed previously. However, *Pax8* has been predicted as a target gene activated via the *Shh* signaling pathway in oncogenesis (Harter et al. 2015). During the development of central nervous system, *Foxj1* expression is regulated by the *Shh* signaling pathway and alters the response of cells

to Shh signaling (Cruz et al. 2010). Although further studies are needed to determine the molecular relationship between Pax8, Foxj1, and Hh signaling-related components, the significant downregulation of the Ptch1 and Smo transcripts in the infundibulum of MRL/lpr mice at 6 months of age may be related to the abnormal pathology of the ciliated epithelium. The alternation of Gli2 localization in the infundibulum of MRL/lpr mice compared to other strains would relate to the downregulation of Ptch1 and Smo transcripts and the disruption of Hh signaling

Aged MRL/lpr mice show autoimmune disease-prone phenotypes in systemic organs, including the oviductal infundibulum with infiltration of autoreactive immune cells because of a mutation in the Fas cell surface death receptor (*Fas*) gene (Andrews et al. 1978; Watanabe-Fukunaga et al. 1992; Hosotani et al. 2020). MRL/lpr at 3 months of age showed expression of *Ptch1*, *Smo*, and *Gli1-3* in the infundibulum, and thus specific genetic alterations in the MRL/lpr strain do not cause significant downregulation of Hh signaling-related components. In contrast, alterations in Hh signaling transduction were reported to trigger immunological modulation. In the skin of atopic dermatitis mouse models and the central nervous system with autoimmune neuroinflammation, Shh signaling suppresses immune reactions via T-regulatory cell signaling and reduces tissue pathology and disease severity (Papaioannou et al. 2019; Benallegue et al. 2021). In adult intestinal mesenchyme, Shh and Ihh act as anti-inflammatory epithelial modulators and modulate tolerogenic versus proinflammatory signaling (Zacharias et al. 2010). Therefore, significant

downregulation of *Ptch1* and *Smo* transcripts may interact with severe autoimmune inflammation in the oviductal lamina propria of MRL/lpr mice at 6 months of age (Hosotani et al. 2020). Furthermore, the deteriorated supply of fresh Hh signaling-related components in the infundibulum of MRL/lpr mice at 6 months of age may be related to abnormal homeostasis of the ciliated epithelium. The damages on ciliary morphofunction caused by variations in levels of inflammatory cytokines in the infundibulum of MRL/lpr mice (Hosotani et al. 2020) are thought to reflect the physiological damage of ciliated epithelium of infundibulum caused by inflammatory follicular fluid (Palma-Vera et al. 2017), in terms of inflammatory cytokines altering the morphofunction of the ciliated epithelium of infundibulum. Therefore, the downregulation of Hh signaling-related components caused by the inflammatory molecules would also relate to the turnover of the infundibulum epithelium under physiological conditions.

We observed a difference in the molecular expression between the infundibulum and ampulla/isthmus. Epithelial cells in the distal and proximal parts of the oviducts are from intrinsically different lineages and are maintained separately (Ford et al. 2020). The properties and populations of Pax8⁺ epithelial cells differ between the distal and proximal parts; the infundibulum shows a low population of Pax8⁺ secretory cells (Ford et al. 2020). Foxj1 upregulation in the infundibulum compared to in the proximal part reflects greater activation of ciliogenesis and a larger population of ciliated epithelial cells in the infundibulum. Hh signaling-related components

were transcribed in the proximal part, despite the sparse presence of cilia. In contrast, correlation analysis indicated that Gli1 is involved in Hh signaling in the proximal part and that Gli1 and Gli3 are involved in epithelial homeostasis. Further investigations are required to reveal the effects of the difference in molecular expression patterns among oviductal parts.

In summary, we propose the different mechanism of Hh signaling transduction between in healthy and autoimmune disease conditions (Figure 8a and b). We propose that homeostasis of ciliated epithelium in the infundibulum is regulated not only by Wnt/ β -catenin and Notch signaling, but also possibly by Hh signaling. In addition, alterations in Hh signal transduction related to the abnormal immune condition in the oviduct of MRL/lpr disrupts the epithelial morphology in the infundibulum, resulting in oocyte pick-up dysfunction. Further functional study on the change of the epithelial morphology in infundibulum under the experimental manipulation of Hh signaling would strengthen the role of Hh signaling in the epithelial homeostasis in oviducts. Our results improve the understanding of the physiology and pathology of mammalian female reproductive function.

Author contributions

Conceptualization: M.H. and O.I.; Methodology: M.H., O.I., Ta.N., M.A.M, Te.N. and Y. H.;
Validation: M.H., O.I., and T.N.; formal analysis: M.H. and O.I.; Investigation: M.H., O.I., Ta.N.
and M.A.M.; Resources: M.H., O.I., and M.A.M.; Data curation: M.H.; Writing - original draft:
M.H. and O.I.; Writing - review & editing: M.H., O.I., T.W., and Y.K.; Visualization: M.H. and
O.I.; Supervision: M.H. and Y.K.; Project administration: O.I. and YK; Funding acquisition: M.H.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical approval

Animal experimentation was approved by the School of Veterinary Medicine, Rakuno Gakuen
University (approval no. VH19A6). Animals were handled in accordance with the Guide for the
Care and Use of Laboratory Animals, Rakuno Gakuen University, Japan.

Data, material, and/or code availability

The data that support the findings of this study are available from the corresponding author
upon reasonable request.

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FIGURE LEGENDS

Fig. 1 Localization of Pax8 and Foxj1 in the oviductal infundibulum. IHC of Pax8 (a-c and a'-c'), Foxj1 (d-f and d'-f'), and negative control (g) are shown. Bar = 25 μ m. Black arrows: immunoreactive positive epithelial cells; red arrowheads: expelled non-ciliated epithelial cells from the epithelium. (h) The percentage of expelled non-ciliated epithelial cells from the epithelium in distal parts of oviducts. There were no significant differences between ages at the same strains (Student's *t*-test, $P < 0.05$) and among strains at the same age (Tukey's test, $P < 0.05$). (i) The percentage of Foxj1 positive expelled non-ciliated epithelial cells from the epithelium distal parts of oviducts. *: Significant differences between 3 and 6 months of age at the same strains and same part (Student's *t*-test, $P < 0.05$). B&M: Significant differences between B6 or MRL/+ mice at the same age (Tukey's test, $P < 0.05$). B6 = C57BL/6N, MRL/+ = MRL/MpJ, MRL/lpr = MRL/MpJ-*Fas*^{lpr/lpr}

Fig. 2 Expression levels of *Pax8* (a) and *Foxj1* (b) in the oviduct. Data are the mean $\pm$ s.e. *: Significant differences between 3 and 6 months of age at the same strains and same part (Student's *t*-test, $P < 0.05$). #: Significant differences between proximal and distal parts of oviducts from mice of the same strains and age (Student's *t*-test, $P < 0.05$). B&M: Significant differences between B6 or MRL/+ mice at the same age (Tukey's test, $P < 0.05$). *Pax8*: Paired box 8; *Foxj1*: Forkhead box

protein J1. B6 = C57BL/6N, MRL/+ = MRL/MpJ, MRL/lpr = MRL/MpJ-*Fas*^{lpr/lpr}

Fig. 3 Localization of hedgehog signaling-related proteins in the oviductal infundibulum revealed by immunofluorescence. Localization of *Ptch1* (a-a''''', b-b''''', and c-c'''''), *Smo* (d-d''''', e-e''''', and f-f'''''), *Gli2* (g-g''''', h-h''''', and i-i'''''), and negative control (j-j'') are shown. Bar = 25 μ m. Arrows: immunoreactive positive cells in the epithelium. B6 = C57BL/6N, MRL/+ = MRL/MpJ, MRL/lpr = MRL/MpJ-*Fas*^{lpr/lpr}

Fig. 4 Expression levels during estrous cycle of hedgehog signaling-related genes in the oviduct of C57BL/6N at 3 months of age. Expression levels of *Ptch1* (a), *Smo* (b), *Gli1* (c), *Gli2* (d), and *Gli3* (e) are shown. Data are the mean $\pm$ s.e. *: Significant differences between 3 and 6 months of age at the same cycle and same part (Student's *t*-test, $P < 0.05$). #: Significant differences between proximal and distal parts of oviducts from mice of the same cycle and age (Student's *t*-test, $P < 0.05$). There were no significant differences among estrous cycles at the same age and part (Tukey's test, $P < 0.05$). P: proximal part; D: distal part 3m: 3 months of age, 6m: 6 months of age. *Ptch1*: Patched-1, *Smo*: Smoothened, *Gli1-3*: GLI family zinc finger 1–3.

Fig. 5 Expression levels of hedgehog signaling-related genes in the oviduct. Expression levels of

Ptch1 (a), *Smo* (b), *Gli1* (c), *Gli2* (d), and *Gli3* (e) are shown. Data are the mean $\pm$ s.e. *: Significant differences between 3 and 6 months of age for the same strains and same part (Student's *t*-test, $P < 0.05$). #: Significant differences between proximal and distal parts of oviducts from mice of the same strains and age (Student's *t*-test, $P < 0.05$). B&M: Significant differences between B6 or MRL/+ mice at the same age (Tukey's test, $P < 0.05$). *Ptch1*: Patched-1, *Smo*: Smoothed, *Gli1-3*: GLI family zinc finger 1-3. B6 = C57BL/6N, MRL/+ = MRL/MpJ, MRL/lpr = MRL/MpJ-*Fas^{lpr/lpr}*

Fig. 6 Expression of transcriptions of hedgehog in ovarian granulosa cells (a-a'', c-c'', and e-e'') and epithelial cells of the oviductal infundibulum (b-b'', d-d'', and f-f'') revealed by *in situ* hybridization. Negative controls are shown in (g and g'). Brown dots indicate positive reactions to *in situ* hybridization. Bar = 25 μ m. Arrows: reaction-positive cells in the oviductal infundibulum. B6 = C57BL/6N; MRL/lpr = MRL/MpJ-*Fas^{lpr/lpr}*

Fig. 7 Expression levels of hedgehog genes in the oviduct of B6 at 3 months of age (a and d), MRL/+ at 3 months of age (b and e), and MRL/lpr at 3 months of age (c and f). Data are the mean $\pm$ s.e. *: Significant differences between proximal and distal parts of oviducts from mice of the same strains and age (Student's *t*-test, $P < 0.05$). 3: Significant differences between 3 and 6 months

505 of age in mice of the same strains and from the same part (Student's *t*-test, $P < 0.05$). B&M:
506 Significant differences between B6 or MRL/+ mice at the same age (Tukey's test, $P < 0.05$). B6 =
507 C57BL/6N, MRL/+ = MRL/MpJ, MRL/lpr = MRL/MpJ-*Fas*^{lpr/lpr}

508

509 **Fig. 8** Estimated modulation mechanisms of hedgehog signaling in the oviductal infundibulum
510 between healthy (a and a') and autoimmune disease conditions (b and b'). In healthy condition,
511 hedgehog signaling transduced by Ihh released by the ovarian granulosa cells, which is received
512 by Ptch1 and Smo localized on the motile cilia of the oviductal infundibulum. The hedgehog
513 signaling mediated by Ptch1, Smo, and Gli2 in the ciliated epithelial cells would lead the constant
514 transcription and protein production of Ptch1 and Smo and maintain the proliferation of Pax8
515 positive secretory cells and differentiation of those into Foxj1 positive healthy ciliated epithelial
516 cells in the oviductal infundibulum. In autoimmune disease condition, the declined levels of Ihh
517 production and transcription of Ptch1 and Smo causes the modulation of hedgehog signaling
518 transduction, which resulted in the disturbances in the homeostasis of ciliated epithelium of the
519 oviductal infundibulum and the appearance of strongly Foxj1 positive ciliated epithelial cells
520 which are morphologically abnormal. CEC: ciliated epithelial cell, SC: secretory cell, NCEC: non
521 ciliated epithelial cell, GCs: granulosa cells, Foxj1: forkhead box J1, Pax8: paired box 8, Hh:
522 Hedgehog, Ihh: indian hedgehog, Ptch1: Patched-1, Smo: Smoothened, Gli2: GLI family zinc

523 finger 2.

Table 1. Primary antibody information in immunostaining.

Anti gen	Cat. No	Sourc e	Ho st	Dilu tion	Antigen retrieval reagent	Blocking serum	Secondary antibody for IF (1:300)	Biotinylated secondary antibody for IHC
Gli2	-	Novus	Rabbit	1:200	20 mM tris-HCl (pH 9.0)	10% goat normal serum	Goat anti-rabbit IgG H&L	-
		Biologicals LLC., Littleton, CO, USA					CF568, 20103-1 (Biotium, Inc., Hayward, CA)	
Ptch1	MAB 41051	R&D Systems Inc., Minneapolis, MN, USA	Rabbit	1:200	0.1% pepsin	10% goat normal serum	Goat anti-rat IgG H&L	-
							CF568, 20096-1 (Biotium, Inc., Hayward, CA)	
Smo	GTX 60154	GeneTex Inc., Irvine, CA, USA	Rabbit	1:200	0.1% pepsin	10% goat normal serum	Goat anti-rabbit IgG H&L	-
							CF568, 20103-1 (Biotium, Inc., Hayward, CA)	
Tubulin- α	MS-581-R7	Thermo Scientific, Waltham, MA, USA	Mouse	Undiluted	No need	10% goat normal serum	Goat anti-mouse IgG H&L CF488A, 20018-1 (Biotium, Inc., Hayward, CA)	-

		MA, USA							
		Invitrogen, Carlsbad, CA, USA	M ouse se	1:50 0	10 mM citrate buffer (pH 6.0)	10% rabbit normal serum	-		Rabbit anti-mouse IgG+IgA+IgM antibody, 426031, undiluted (Nichirei, Tokyo, Japan)
		Biocal, Mediocal, Pacheco, CA, USA	M ouse se	1:10 0	20 mM tris- HCl (pH 9.0)	10% rabbit normal serum	-		Rabbit anti-mouse IgG+IgA+IgM antibody, 426031, undiluted (Nichirei, Tokyo, Japan)

525 IF: immunofluorescence, IHC: immunohistochemistry.

526 **Table 2. Primer list used for quantitative PCR analysis.**

Genes	Accession Number	Primer Sequence (5'-3') F: Forward, R: Reverse		Product size (bp)	Annealing temp. (°C)
<i>Actb</i>	NM_007393.5	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAAA		165	60
<i>Dhh</i>	NM_007857.5	F: TTGGCACTCTTGGCACTATCT R: CTTTGCAACGCTCTGTCATC		277	60
<i>Foxj1</i>	NM_008240.3	F: ACTATGCCACCAACCCACA R: GGATGGAATTCTGCCAGGT		171	60
<i>Gli1</i>	NM_010296.2	F: CGACCTGCAAACCGTAATC R: CTTGCCAACCATCATATCCA		289	60
<i>Gli2</i>	NM_001081125.1	F: TGGAGAAGAAAGAAGCCAAGAG R: TCATGTCAATCGGCAAAGG		159	60
<i>Gli3</i>	NM_008130.3	F: CCTGCTCCAACATTTC AAC R: CTTGACTAGGGTTGTTTCCTTCC		240	60

		F: CCTCTTGCCTACAAGCAGTTC		
<i>Ihh</i>	NM_010544.3		214	60
		R: AGATGGCCAGTGAGTTCAGAC		
		F: AAGCATCGACTCACAGAGCA		
<i>Pax8</i>	NM_011040.4		285	60
		R: GAATGAGGATCTGCCACCAC		
		F: CCATGACAAAGCCGACTACA		
<i>Ptch1</i>	NM_001328514.1		293	60
		R: GGAAGACTGCGCACACTAGAA		
		F: CAAGTACGGCATGCTGGCTC		
<i>Shh</i>	NM_009170.3		254	52
		R: AAGGTGAGGAAGTCGCTGTA		
		F: GTCTCTGCACGCTCTTCACA		
<i>Smo</i>	NM_176996.4		269	60
		R: CCAGACTACTCCAGCCATCAA		

527 *Dhh*: desert hedgehog, *Foxj1*: forkhead box J1, *Gli1-3*: GLI family zinc finger 1–3, *Ihh*: Indian
528 hedgehog, *Pax8*: paired box 8, *Ptch1*: Patched 1, *Shh*: sonic hedgehog, *Smo*: smoothened, frizzled
529 class receptor.

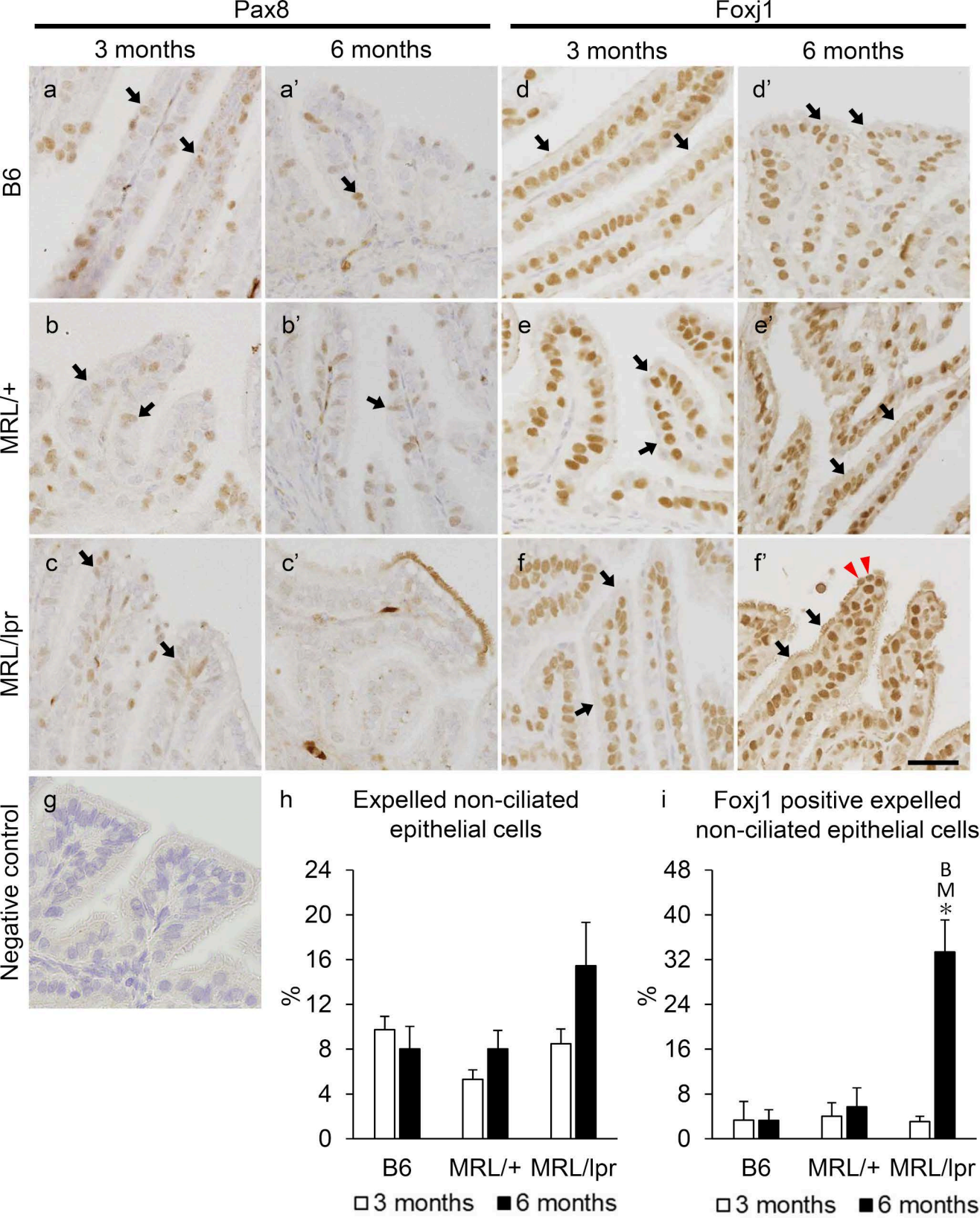
530 **Table 3. Spearman's correlation coefficient (ρ) between hedgehog signaling-related genes**
531 **expression and secretory/ciliated epithelial cells marker expression in the oviduct of mice at**
532 **3 months of age.**

		Parameters					
		Hedgehog receptor gene expression	Secretory and ciliated epithelial cells marker expression		Hedgehog receptor gene expression	Secretory and ciliated epithelial cells marker expression	
		at proximal part			at distal part		
		<i>Ptch1</i>	<i>Pax8</i>	<i>Foxj1</i>	<i>Ptch1</i>	<i>Pax8</i>	<i>Foxj1</i>
<i>Ptch1</i>	ρ	1	0.55***	0.38**	1	0.63***	0.62***
	<i>P</i>	-	<0.0001	0.0071	-	<0.0001	<0.0001
<i>Smo</i>	ρ	0.60***	0.62***	0.42**	0.48***	0.48***	0.59***
	<i>P</i>	<0.0001	<0.0001	0.0026	0.0007	0.0007	<0.0001
<i>Gli1</i>	ρ	0.63***	0.49***	0.20	0.19	0.13	0.23
	<i>P</i>	<0.0001	0.0005	0.1690	0.1981	0.3862	0.1226
<i>Gli2</i>	ρ	0.56***	0.72***	0.69***	0.43**	0.61***	0.69***

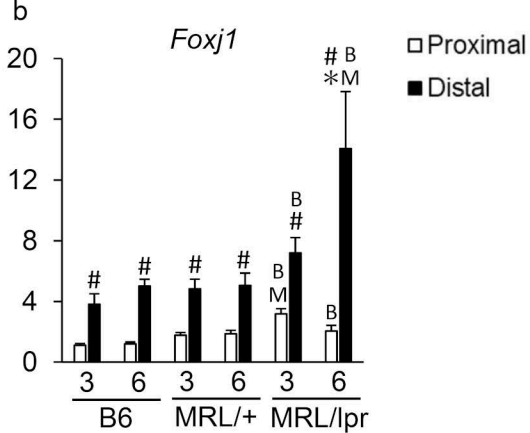
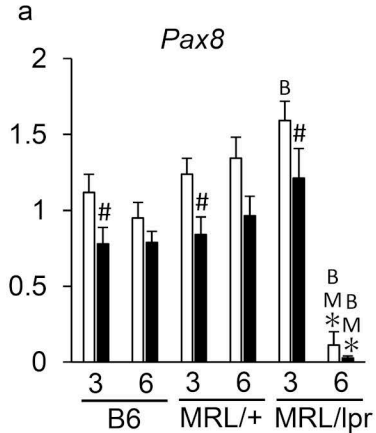
	<i>P</i>	<0.0001	<0.0001	<0.0001	0.0027	<0.0001	<0.0001
<i>Gli3</i>	ρ	0.41**	0.39**	0.35*	-0.30*	-0.09	0.07
	<i>P</i>	0.0038	0.0059	0.0141	0.0431	0.5554	0.6623

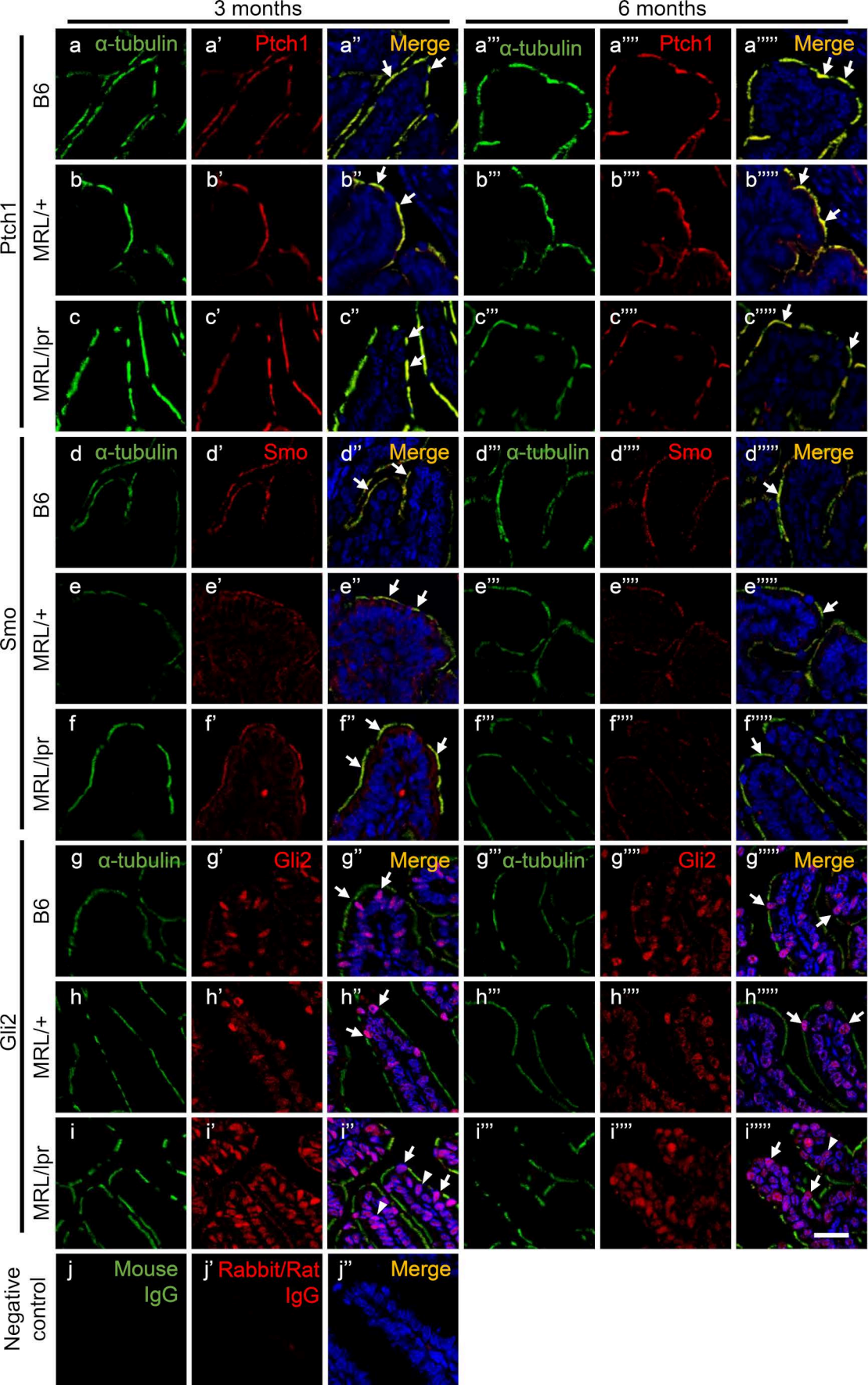
534 *Ptch1*, Patched-1; *Smo*, Smoothed; *Gli1-3*, GLI family zinc finger 1–3, *Pax8*, paired box 8;

535 *Foxj1*, forkhead box J1.

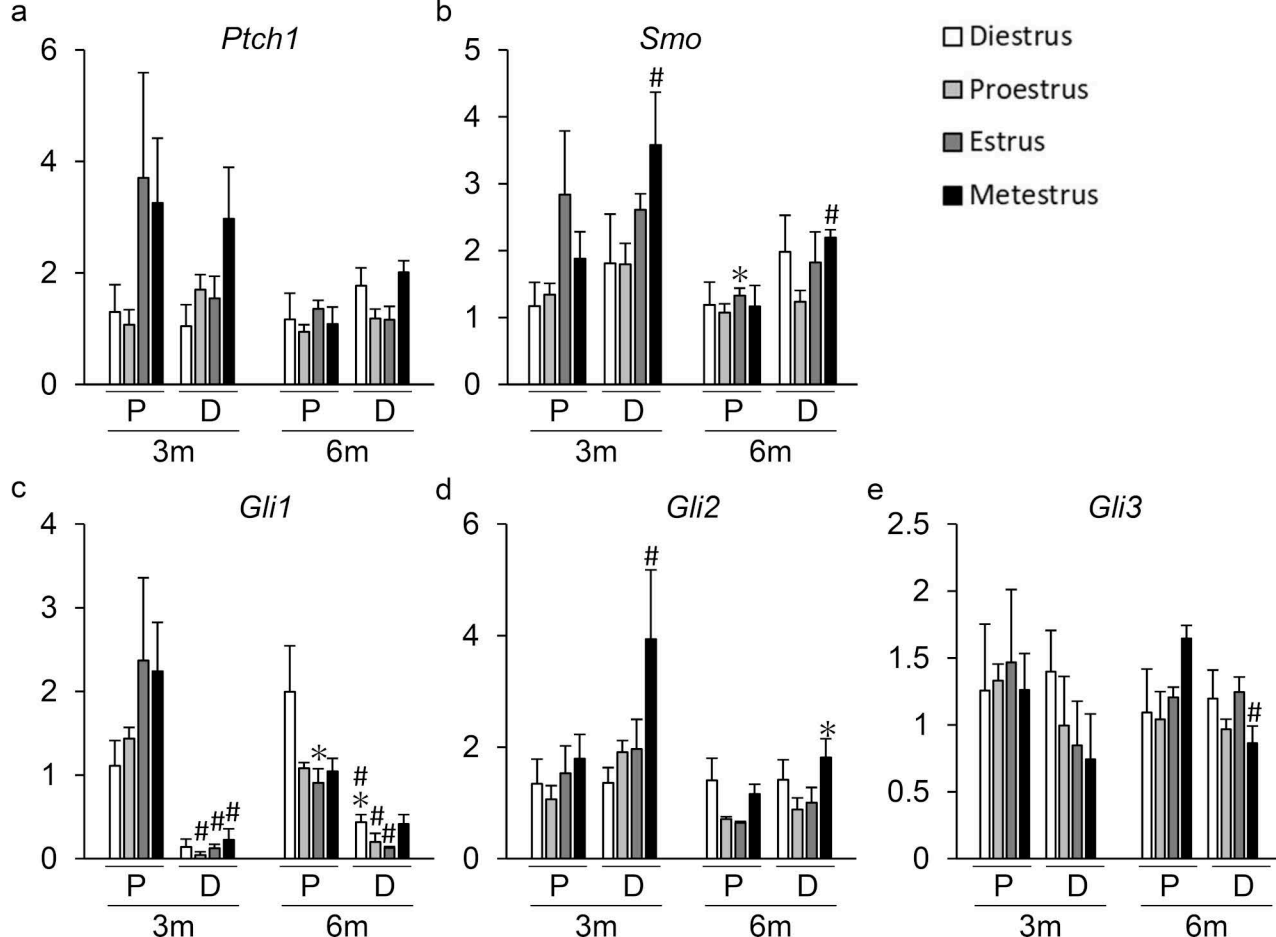


$\Delta\Delta C_t$ vs proximal B6 at 3 months

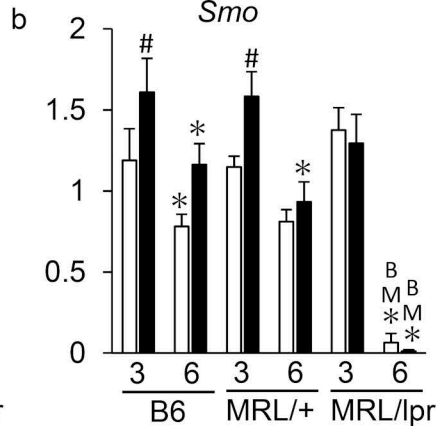
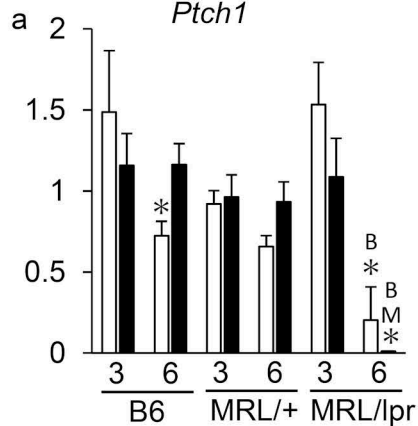




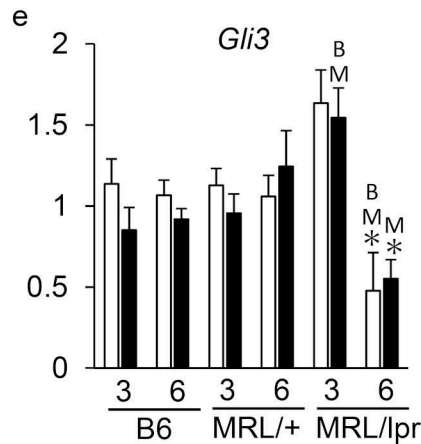
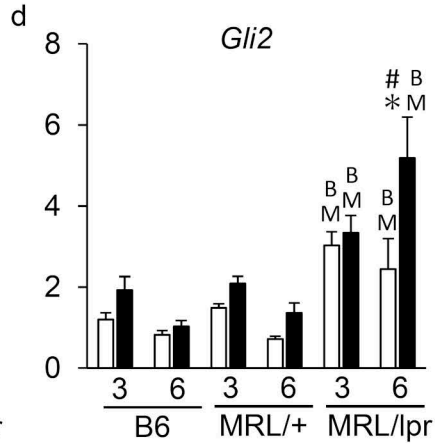
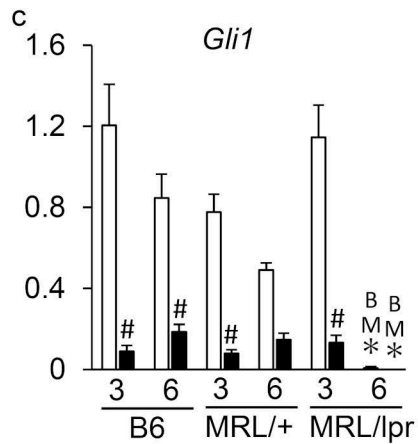
$\Delta\Delta C_t$ vs proximal at 3 months & anestrus

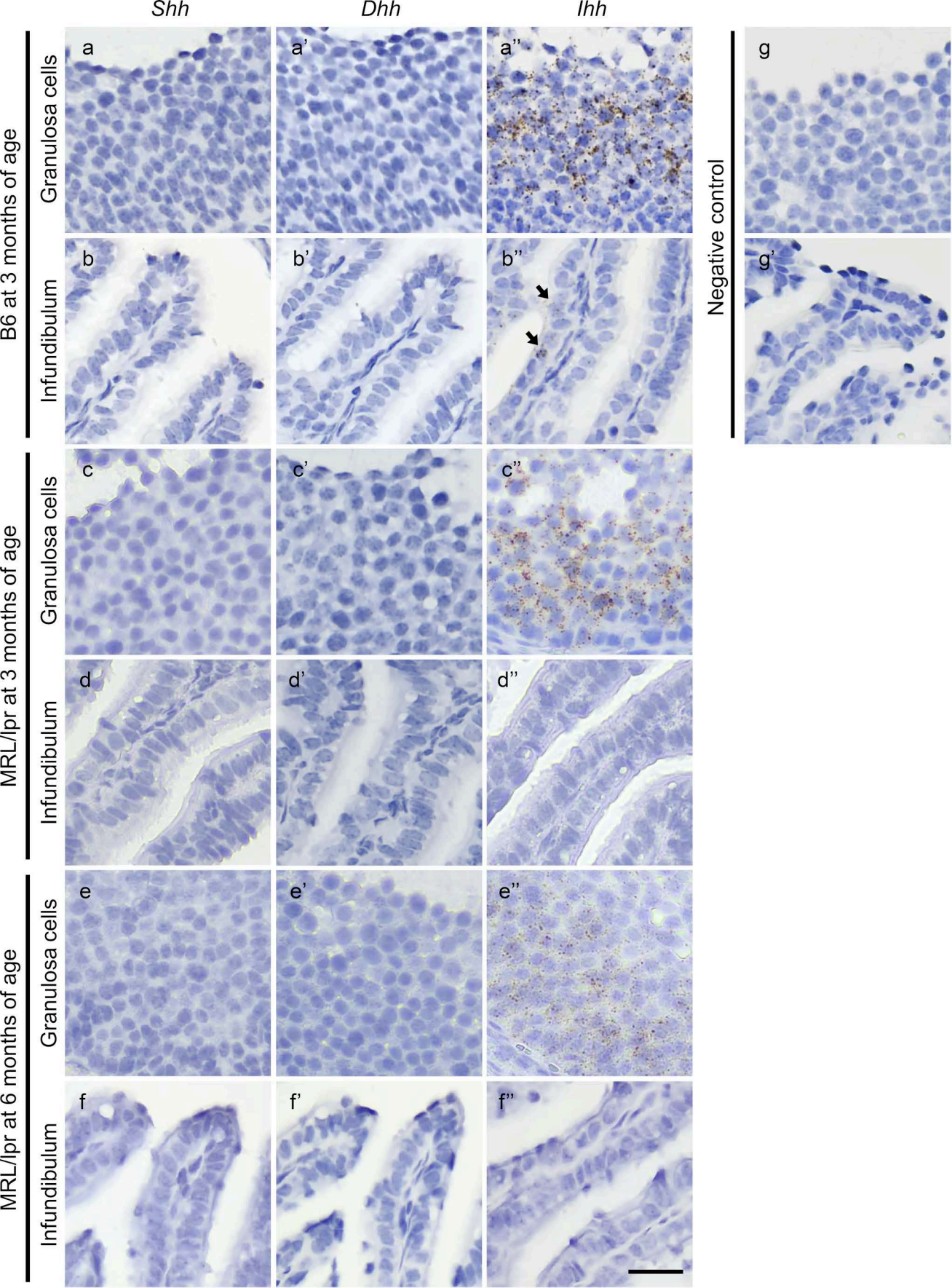


$\Delta\Delta C_t$ vs proximal B6 at 3 months

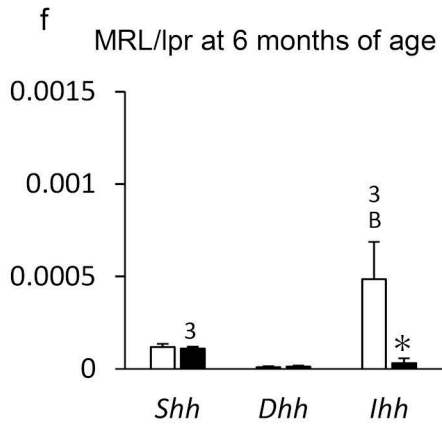
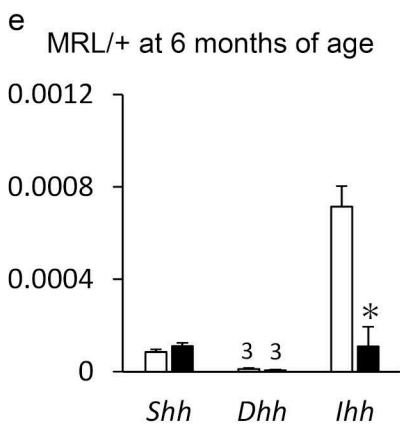
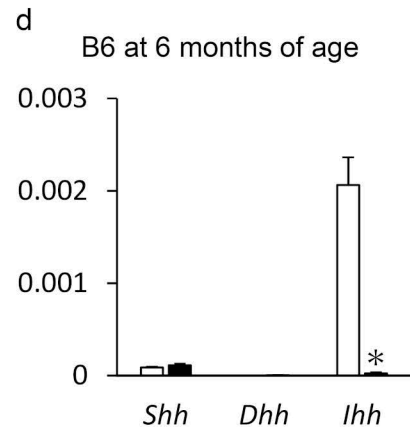
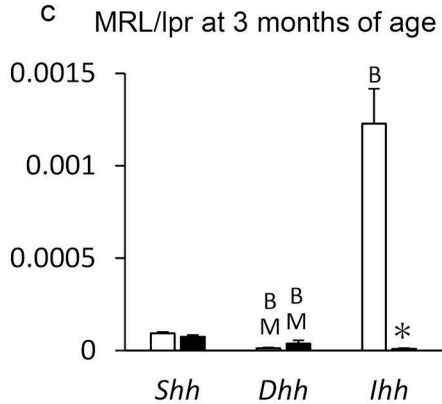
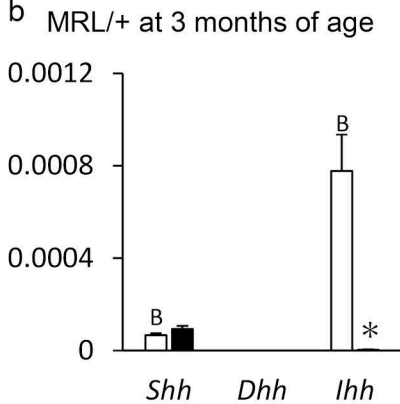
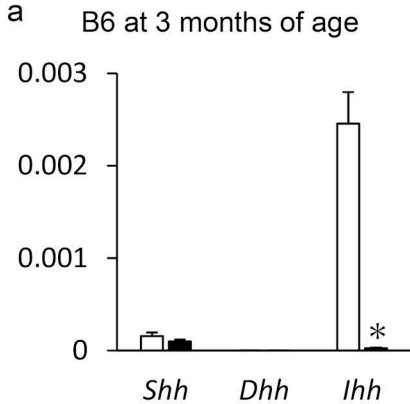


□ Proximal
■ Distal





ΔC_t vs *Actb*



□ Proximal ■ Distal

