



Title	Anatomy and histology of the foramen of ovarian bursa opening to the peritoneal cavity and its changes in autoimmune disease prone mice
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Citation	Journal of Anatomy, 238(1), 73-85 <a href="https://doi.org/10.1111/joa.13299">https://doi.org/10.1111/joa.13299</a>
Issue Date	2021-01
Doc URL	<a href="http://hdl.handle.net/2115/84017">http://hdl.handle.net/2115/84017</a>
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1 **Anatomy and histology of the foramen of ovarian bursa opening to the peritoneal cavity and its changes**  
2 **in autoimmune disease-prone mice**

3 **Short running:** The foramen of ovarian bursa in mouse

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## Abstract

The ovarian bursa is a small peritoneal cavity enclosed by the mesovarium and mesosalpinx, which surrounds the ovaries and oviductal infundibulum in mammals. The ovarian bursa is considered as the structure facilitating the transport of ovulated oocytes into the oviduct. Our previous study revealed reduced oocyte pick-up function in the oviduct of lupus-prone MRL/MpJ-Fas<sup>lpr/lpr</sup> mouse, suggesting the possibility of an escape of ovulated oocytes into the peritoneal cavity, despite the presence of an almost complete ovarian bursa in the mouse. In this study, we revealed anatomical and histological characteristics of the ovarian bursa in C57BL/6N, MRL/MpJ and MRL/MpJ-Fas<sup>lpr/lpr</sup> mice. All strains had the foramen of ovarian bursa (FOB), with a size of approximately 0.04 to 0.12 cm<sup>2</sup>, surrounded by the ligament of ovarian bursa (LOB), which is part of the mesosalpinx. The LOB was partially lined with the cuboidal mesothelial cells and consisted of a thick smooth muscle layer in all strains. In 6-month-old MRL/MpJ-Fas<sup>lpr/lpr</sup> mice, in which the systemic autoimmune abnormality deteriorated and oocyte pick-up function was impaired, the size of the FOB tended to be larger than that of other strains. Additionally, in MRL/MpJ-Fas<sup>lpr/lpr</sup> mice at 6 months of age, there was infiltration by numerous immune cells in the mesosalpinx suspending the isthmus; however, the LOB prevented severe inflammation and showed deposition of collagen fibers. These results not only indicate that the FOB is a common structure within mice, but also imply the physiological function of the LOB and its role in maintaining the microenvironment around the ovary, as well as regulating healthy reproduction.

**Keywords:** ovarian bursa; oviduct; mesosalpinx; 3D morphometry; reproduction

## 40 **Introduction**

41 The ovaries originate from the gonadal primordium, located in the lumbar region on the medial surface of  
42 mammalian mesonephros (König et al., 2009). Moreover, the oviducts and uterus develop from the Müllerian  
43 ducts (Yamamoto et al., 2018) and play crucial roles in female reproduction. The ovaries and oviducts are  
44 suspended within the mesovarium and mesosalpinx, respectively. These are the cranial parts of the broad ligament,  
45 ligamentum latum uteri, which is the common double-folded suspension of the mammalian female genital tract  
46 to the abdominal wall (König et al., 2009). The ovarian ligaments and the proper ligament of the ovary,  
47 ligamentum ovarii proprium, connect each ovary to the lateral side of the uterus (Craig and Billow, 2018). The  
48 mesovarium, mesosalpinx, and the proper ligament of the ovary enclose a small peritoneal cavity, termed the  
49 ovarian bursa, bursa ovarica, which surrounds the ovary and oviductal infundibulum (König et al., 2009).

50 The anatomical characteristics of the ovarian bursa, as well as ovulation rates, vary depending on the animal  
51 species. In cows, ovulation produces one oocyte at a time (Peters and McNatty, 1980), and the mesosalpinx  
52 surrounds the ovary like a mantle and forms a voluminous ovarian bursa with a wide cranio-ventromedial opening  
53 (Budras and Budras, 2003). In mares, ovulation produces one oocyte at a time (Ginther et al., 2001), and the ovary  
54 is too large to be located within the ovarian bursa (König et al., 2009). In female dogs, multiple ovulations produce  
55 about 5 to 7 oocytes at a time (Miranda et al., 2018), and the ovarian bursa completely encompasses the ovary  
56 within the foramen of ovarian bursa (FOB, *foramen bursae ovaricae*), which is a narrow slit-like opening to the  
57 peritoneal cavity (König et al., 2009). In mice, the ovulation rate is about eight at once, although the ovulation  
58 rate varies depending on the mice strain (Peters and McNatty, 1980); the ovaries are completely surrounded by

59 the ovarian bursa, which has a small peritoneal opening (Wimsatt and Waldo, 1945). Finally, female human have  
60 no bursal structure around the ovaries (Beck, 1972; Ng and Barker, 2015).

61 The ovarian bursa is thought to play a role in preventing ovulated oocytes from escaping into the peritoneal  
62 cavity, thus facilitating the transport of ovulated oocytes into the oviduct and assisting effective fertilization  
63 (Zhang et al., 2013). Furthermore, surgical removal of the ovarian bursa surrounding the ovary of rodents leads  
64 to a reduction in the number of oocytes picked-up by the oviductal infundibulum within the oviduct (Vanderhyden  
65 et al., 1986; Kaufman et al., 2010). Based on the aforementioned anatomy and function of the murine ovarian  
66 bursa, it has been suggested that the oocytes produced in the ovaries rarely escape into the peritoneal cavity in  
67 mice.

68 In our previous work, we found that autoimmune disease-prone MRL/MpJ-*Fas*<sup>lpr/lpr</sup> (MRL/lpr) mice suffer a  
69 reduction in oocyte pick-up by the oviductal infundibulum, resulting due to the progression of their systemic  
70 autoimmune conditions (Hosotani et al., 2018). Furthermore, we showed that the ruptured follicles and corpora  
71 hemorrhagica counts in whole-serial-sectioned ovaries, accounting for ovulated oocytes, exceeded the count of  
72 cumulus-oocyte complexes in the oviductal ampulla picked up by the oviductal infundibulum of MRL/lpr mice  
73 at 6 months of age with severe autoimmune abnormalities (Hosotani et al., 2018). This previous study unraveled  
74 the possibility that the oocytes which progress into the ovarian bursa of mice are expelled into the peritoneal  
75 cavity through the small peritoneal opening in the ovarian bursa. However, only a few anatomical and histological  
76 studies of the murine ovarian bursa are available to confirm this hypothesis; hence, further investigations are  
77 needed.

78 In the present study, we visualized and analyzed the structure of the ovarian bursa of three murine models:  
79 C57BL/6N (B6), MRL/MpJ (MRL/+), and MRL/lpr at 3 and 6 months of age. C57BL/6N (B6) is the most widely  
80 used of inbred mouse strain (Bryant, 2011), MRL/MpJ (MRL/+) is the parent and control strain for MRL/lpr  
81 (Heydemann, 2012), and the MRL/lpr strain is a systemic autoimmune disease-prone model which has an oocyte  
82 pick-up disorder, whose disease deteriorates at 6 months of age compared to 3 months of age (Hosotani et al.,  
83 2018). We found that all strains had the FOB within the mesosalpinx connecting oviduct and uterus. We also  
84 report the histological characteristics of the mesosalpinx surrounding the FOB, which is named the ligament of  
85 FOB (LOB). Moreover, we show that the LOB differs from other parts of the mesosalpinx, as the LOB is partially  
86 lined with cuboidal mesothelial cells and consists of a thick smooth muscle layer in all strains. In 6-month-old  
87 MRL/lpr mice with severe autoimmune disease, although the LOB prevented the infiltration of immune cells, it  
88 showed fibrosis; moreover, the FOB size in some of these mice was markedly larger than that of other strains and  
89 3-month-old MRL/lpr mice. The results of our study provide insight on the anatomy, histology, reproductive  
90 biology, and experimental technology of the mouse.

## 92 **Materials and Methods**

### 93 *Animals*

94 Animal experimentation was approved by the Institutional Animal Care and Use Committee of the Graduate  
95 School of Veterinary Medicine, Hokkaido University (Approval No.15-0079), and the School of Veterinary  
96 Medicine, Rakuno Gakuen University (Approval No. VH19A6). Animals were handled in accordance with the

97 Guide for the Care and Use of Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University,  
98 Japan (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International),  
99 and with the Guide for the Care and Use of Laboratory Animals, Rakuno Gakuen University, Japan. Female B6,  
100 MRL/+, and MRL/lpr mice at 3 and 6 months of age were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka,  
101 Japan). Previous studies reported that autoimmune disease is severely exacerbated in female MRL/lpr mice at 6  
102 months of age compared to its severity at 3 months of age (Hosotani et al., 2018, 2020). The mice were housed  
103 in groups within plastic cages at 18 - 26°C, under a 12 h light/dark cycle and had free access to a commercial diet  
104 and water. The estrous cycle of each mouse was confirmed by monitoring vaginal smears. All mice were  
105 euthanized by either severing of carotid artery or cervical dislocation, under deep anesthesia using a mixture of  
106 medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). The spleen was collected from  
107 mice and the weight was measured.

### 109 *Stereomicroscopical and histological observation of mice female reproductive tract*

110 The female reproductive tract, including ovaries, oviducts, and a cranial part of the uterus were collected  
111 from mice. The morphology of these organs were kept in 0.01M phosphate buffered saline (PBS) and were  
112 observed under a stereo microscope. After observation, the organs were fixed with 4% paraformaldehyde (PFA)  
113 at 4°C overnight, embedded in paraffin, and cut into 3 µm-thick sections for immunohistochemistry and Masson's  
114 trichrome staining (MT) investigations.



## **India ink injection into the ovarian bursa**

The female reproductive tract, including ovaries, oviducts, and a cranial part of the uterus, were collected from mice. A total of 10 to 20  $\mu$ l India ink was injected into the ovarian bursa by inserting a glass capillary or 35 gauge needles, and the leakage of India ink through the FOB was observed.

## ***Ultrastructural analysis of mice female reproductive tract***

The female reproductive tract, including ovaries, oviducts, and a cranial part of the uterus were removed from mice during the estrus cycle and fixed using a fixing solution, containing 2.5% glutaraldehyde and 2% PFA. After six washes in 0.1 M phosphate buffer (PB), these organs were post-fixed with 1% osmium tetroxide in 0.1 M PB for 2 h at room temperature. After six washes in distilled water, the specimens were subjected to conductive treatment by 5% BEL-1 (Nisshin EM Co. Ltd., Tokyo, Japan) in 70% ethanol for 2 h at room temperature. Specimens were dried completely and examined using a S-2460N scanning electron microscope (Hitachi, Tokyo, Japan). Samples were sputter coated with gold using the ion-sputter E102 (Hitachi, Tokyo, Japan).

## ***Area measurement of the opening of ovarian bursa reconstructed three-dimensionally***

The ovaries, oviducts and the cranial part of the uterus were collected from mice during estrus. These organs were fixed with 4% PFA and kept at 4°C overnight. They were then embedded in paraffin and cut into 12  $\mu$ m-thick whole serial sections. The serial hematoxylin and eosin-stained sections were used for both the histological observation and the three-dimensional (3D) reconstruction of the female reproductive organs. The 3D

135 reconstruction was processed using Fiji software, which is an image processing package of ImageJ (National  
136 Institutes of Health, Bethesda, Maryland, USA), and Image Pro software (Media Cybernetics Inc., Rockville,  
137 Maryland, USA). The alignment of each pictured section in whole serial sections was adjusted by the Register  
138 Virtual Stack Slices plugin provided in Fiji (National Institutes of Health, Bethesda, Maryland, USA). Based on  
139 these aligned 2D pictures of the female reproductive tract, 3D geometrical models of female reproductive tracts  
140 were created using the Image Pro software (Media Cybernetics Inc., Rockville, Maryland, USA). The peritoneal  
141 side of the FOB observed in these 3D models was measured using Image Pro software (Media Cybernetics Inc.,  
142 Rockville, Maryland, USA).

#### 144 ***Immunohistochemistry***

145 The 3  $\mu\text{m}$ -thick sections of female reproductive organs of mice during estrus were incubated in 20 mM tris-  
146 HCl (pH 9.0) (for B220, B cell marker; CD3, T cell marker; Foxp3, regulatory T cell marker), or 10 mM citrate  
147 buffer (pH 6.0) (for Iba1, macrophage marker; Lyve1, lymphatic vessel marker; alpha smooth muscle actin  
148 ( $\alpha\text{SMA}$ ), smooth muscle cell marker) for 15 min at 110°C. Sections were then soaked in methanol, containing  
149 0.3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Sections that were blocked using 10% normal goat serum for 60 min at room  
150 temperature were incubated at 4°C overnight with rat anti-B220 (1:1600, Cedarlane, Ontario, Canada), rabbit  
151 anti-CD3 (ready to use, Nichirei Bioscience Inc., Tokyo, Japan), rabbit anti-Iba1 (1:1200, FUJIFILM Wako Pure  
152 Chemical Co., Ltd., Osaka, Japan), rat anti-Foxp3 (1:100, Thermo Fisher Scientific K.K., Tokyo, Japan), rabbit  
153 anti-Lyve1 (1:500, AdipoGen Inc., San Diego, California, USA) or rabbit anti- $\alpha\text{SMA}$  (1:3000, Abcam CO.,

154 Cambridge, UK). After three washes in 0.01 M PBS, sections were incubated with biotin-conjugated goat anti-  
155 rabbit IgG (SABPRO Kit, Nichirei Bioscience Inc.), or goat anti-rat IgG antibody (1:150, BioLegend Inc., San  
156 Diego, California, USA) for 30 min, and washed and incubated for 30 min at room temperature, using a  
157 streptavidin-biotin complex (SABPRO Kit, Nichirei Bioscience Inc.). Sections were then incubated with 3, 3' –  
158 diaminobenzidine tetrahydrochloride-H<sub>2</sub>O<sub>2</sub> solution, and lightly stained with hematoxylin.

### 160 *Statistical analysis*

161 Results were expressed as mean ± standard error of the mean (SEM) and statistically analyzed in a non-  
162 parametric manner. Two groups were compared using the Mann-Whitney *U*-test ( $P < 0.05$ ). The Kruskal-Wallis  
163 test was used to compare the three groups; multiple comparisons were performed using Scheffé's method when  
164 significant differences were observed ( $P < 0.05$ ).

### 166 **Results**

#### 167 *The morphology of the peritoneal opening of the ovarian bursa of mice*

168 Under the stereomicroscope, it was found that there is common positional anatomy of the female  
169 reproductive tract among the three strains at both ages, and both the left and right ovaries were located on the  
170 cranial side of the oviducts, folded like a coil connecting to the uterus (Figure 1). Most parts of the ovaries in all  
171 mice were covered by continuous mesovarium and mesosalpinx. Notably, MRL/+ mice at 6 months of age  
172 developed ovarian cysts (Figure 1) (Kon et al., 2007). In the magnified stereomicroscopical observations, the

173 oviducts folded like a coil by the mesosalpinx were clearly observed in the female reproductive tract of all strains.  
174 The coiled oviducts were connected to the cranial part of the uterus by the mesosalpinx, which surrounded the  
175 slit-shaped peritoneal openings in the ovarian bursa. Both the left and right ovaries had peritoneal openings  
176 generally less than 1 mm in length, which were commonly observed at the same position of the female  
177 reproductive tract of all strains at both ages. The slit-shaped peritoneal opening in the ovarian bursa of the mouse  
178 is named the FOB, based on the anatomical vocabulary of the narrow slit-like opening observed in the ovarian  
179 bursa of female dogs (König et al., 2009). The mesosalpinx surrounding the murine FOB is named the LOB  
180 (ligament of ovarian bursa). Some, but not all, of the ovarian bursa of the 6-month-old MRL/lpr mice exhibited a  
181 larger FOB than that of other individuals. Furthermore, the oviductal infundibulum enclosed the ovarian bursa  
182 and was observed from the peritoneal side, as shown in the FOB of the right side ovaries of MRL/lpr mice at 6  
183 months of age in Figure 1. The India ink injected into the ovarian bursa leaked through the FOB in the three  
184 strains at both ages (Figure 2 and Supplementary Movie S1).

185 The ultrastructure of FOB was also observed in all strains (Figure 3). As shown through stereomicroscopical  
186 observations, the FOB in mice was surrounded by the LOB, which was slit-shaped or had a slightly expanded  
187 ellipsoid shape. Furthermore, mesothelial cells lining the LOB in B6 and MRL/+ mice exhibited spherical dome  
188 like morphology (Figure 3, arrowheads in insets). In MRL/lpr mice, the mesothelium of the LOB had a deeply  
189 tangled surface. The ultrastructural differences of the FOB between the left and right sides were not observed.

190 The female reproductive tract reconstructed in 3D by superimposing the images of their whole serial  
191 sections reproduced the morphology of the FOB in the mesothelium (Figure 4A). Furthermore, we measured the

192 peritoneal side area of the FOB (Figure 4B). Although the area had no significant differences among strains at  
193 both 3 and 6 months of age, MRL/lpr mice at 6 months of age tended to have a larger FOB size than other strains  
194 at the same age.

#### 196 *The histology of the LOB of mice*

197 The presence of the FOB in all strains was confirmed in the histological sections shown in Figure 5. The  
198 whole serial sections of the female reproductive tract of mice revealed that the ovarian bursa did not have apertures  
199 that were continuous with the peritoneal cavity, other than the FOB (data not shown). This histological observation  
200 also showed that the FOB was surrounded by the LOB connecting the isthmus or ampulla of the oviducts and the  
201 cranial part of the uterus. The epithelium of the LOB facing the FOB was lined with mesothelial cells, which  
202 were, in part, cuboidal epithelial cells (Figure 5). Significant histological differences in the LOB were not  
203 observed among the strains at both 3 and 6 months of age and the left and right sides.

#### 205 *The distribution of smooth muscle cells and collagen fibers in the LOB of mice*

206 Immunohistochemical analysis identified smooth muscle cells as the cell types that compose the connective  
207 tissue under the epithelium of the LOB (Figure 6). To examine the distribution of the collagen fibers in the LOB,  
208 MT staining was performed (Figure 6, MT). In the LOB of B6 mice at both 3 and 6 months of age, the thick  
209 collagen fibers were distributed underneath the mesothelium and between smooth muscle layers (Figure 6). In the  
210 LOB of MRL/+ mice at 3 and 6 months of age, as well as in MRL/lpr mice at 3 months of age, the distribution of

211 thick collagen fibers was not significant. However, it was observed that the thick and dense aniline blue<sup>+</sup> collagen  
212 fibers were distributed underneath the mesothelium and partially between the smooth muscle layers in the LOB  
213 of MRL/lpr mice, at 6 months of age (Figure 6).

#### 214 215 *The distribution of immune cells in the LOB of mice*

216 MRL/lpr mouse is well known as a severe systemic autoimmune disease model (Kamogawa et al., 2002).  
217 The severe inflammation of immune cells affects ovaries and the oviduct of MRL/lpr mice at 6 months of age  
218 (Otani et al., 2015; Hosotani et al., 2018). In this study, we confirmed that MRL/lpr mice showed more significant  
219 splenomegaly at 3 and 6 months of age compared to that of other strains. We also showed that the splenomegaly  
220 in MRL/lpr mice was exacerbated at 6 months of age (Supplementary Figure S2). To examine the effect of the  
221 inflammation in the LOB of the mice, we performed immunohistochemistry to detect B cells, T cells,  
222 macrophages, and regulatory T cells. In the mesosalpinx folding the isthmus, there was significant infiltration of  
223 immune cells, including B220<sup>+</sup> B cells, CD3<sup>+</sup> T cells, and Iba1<sup>+</sup> macrophages (Figure 7). In MRL/lpr mice at both  
224 3 and 6 months, compared to the other strains, a larger distribution of Foxp3<sup>+</sup> regulatory T cells was observed  
225 (Figure 7). The infiltration of the immune cells was more profound in MRL/lpr mice at 6 months of age than at 3  
226 months of age. However, no significant distribution of B220<sup>+</sup> B cells, CD3<sup>+</sup> T cells, Iba1<sup>+</sup> macrophages, or Foxp3<sup>+</sup>  
227 regulatory T cells in the LOB was observed in any of the mice used in this study (Figure 8). The lymphatic vessels,  
228 visualized by the Lyve1 positive reaction, were distributed directly underneath the LOB, as well as in the  
229 connective tissue of the LOB, in all strains at 3 and 6 months of age. The number of these immune cells and

230 lymphatic vessels in the LOB exhibited no differences among the strains at 3 and 6 months of age.

## 232 **Discussion**

233 Over 70 years ago, Wimsatt and his colleagues reported for the first time that a peritoneal opening generally  
234 appears in the ovarian bursa of B6 and Swiss strain of mice (Wimsatt and Waldo, 1945). However, the rather  
235 small morphology of the FOB, which is rarely observed in the histological section of female reproductive tracts,  
236 led to misinterpretations in several reports which stated that mice have a complete ovarian bursa with no peritoneal  
237 opening, with completely enveloped ovaries (Beck, 1972; Cotchin and Marchant, 1977a, 1977b; Kaufman et al.,  
238 2010; Dixon et al., 2014). This study confirmed and revealed that both B6 and MRL-background strains of mice  
239 have peritoneal openings in the ovarian bursa, indicating that the FOB surrounded by the LOB, which connects  
240 the oviduct and the cranial part of the uterus, is the general female reproductive structure in mice (Figure 9). As  
241 for other rodents, rats also have a small opening in the ovarian bursa to the peritoneal cavity (Kellogg, 1941).  
242 However, in golden hamsters, each ovary has been reported to be enclosed within a complete bursa that is  
243 connected with the oviduct (Cotchin and Marchant, 1977b; Martin et al., 1981; Shinohara et al., 1987).  
244 Interestingly, the monotocous species possess neither a bursa (as in the case of the female human) nor an ovarian  
245 bursa that is strongly connected with the peritoneal cavity (as in the cases of the mare and cow), but polytocous  
246 species possess an almost complete ovarian bursa (as in the cases of the rodents) (Kaufman et al., 2010). The  
247 anatomical characteristics of the mammalian ovarian bursa can provide key information to explain the biological  
248 and evolutionary differences in their reproduction.

249 The almost closed appearance of the murine ovarian bursa has been used for the intrabursal injection technique,  
250 which is a method of topical drug delivery to ovaries by the injection of a solution into the bursal cavity of an  
251 anesthetized animal (Martin et al., 1981; Van der Hoek et al., 2000). It is also used as the method for selective  
252 introduction of genetic information to alter the ovarian surface epithelium (Clark-Knowles et al., 2007; Kaufman  
253 et al., 2010). However, it has been suggested that the ovarian bursa may play an active role in regulating local  
254 fluid homeostasis during the ovulation (Zhang et al., 2013). The intrabursal fluid interchange is thought to be  
255 bidirectional between the peritoneal cavity and the reproductive tract through the FOB. This rationale is based on  
256 the observation that particles of india ink, which was injected into the peritoneal cavity of mice, were found to be  
257 abundantly present in the ovarian bursa and oviduct during the ovulation period (Wimsatt and Waldo, 1945).  
258 Although we examined the FOB and LOB of the mice at estrus in this study, further studies using mice at various  
259 stages in the estrous cycle can help to reveal additional morphological differences between the FOB and LOB.  
260 Furthermore, we found that the LOB possesses a thick, smooth muscle layer, which indicates that the FOB alters  
261 its area depending on the physiological, hormonal, and pathological conditions of the female reproductive tract  
262 by contracting the LOB. Therefore, in order to get accurate results, the selection of an optimal injection time,  
263 while taking into consideration the stage of the estrous cycle and/or light-dark cycle, as well as the consistency  
264 of the injection timing through a series of experiments is important for researchers who perform intrabursal  
265 injections.

266 The ovarian bursa is a key player in maintaining an adaptive ovarian microenvironment for ovulation (Li et  
267 al., 2007). Lymphatic stomata are small openings in lymphatic capillaries on the free surface of the mesothelium



268 (Wang et al., 2010). The ovarian bursa of the golden hamster has lymphatic stomata that connects the bursal cavity  
269 with the lymphatic lumen (Shinohara et al., 1987). It is suggested that the opening area of lymphatic stomata  
270 varies under different fluid pressure and mechanical forces (Li et al., 2007). In addition to these theories, the  
271 muscular structure of the LOB surrounding the FOB provides us with a novel hypothesis that the contraction of  
272 the FOB also plays a role in maintaining the bursal liquid and/or hormonal homeostasis, by discarding the bursal  
273 liquid into the peritoneal cavity.

274 The peritoneum is composed of an extensive squamous or cuboidal monolayer of mesothelial cells that rests  
275 on the fibrous connective tissue underneath (Yung et al., 2006; Isaza-Restrepo et al., 2018). The cuboidal  
276 mesothelial cells appear in the septal folds of the mediastinal pleura, liver, and spleen, and are in a metabolically  
277 active state (Mutsaers, 2002). The peritoneum facilitates immune induction, modulation, and inhibition, as the  
278 mesothelial cells are capable of recognizing pathogens and tissue damage, and initiating inflammatory responses  
279 through antigen presentation, cytokine production, and interaction with immune cells, such as macrophages  
280 (Isaza-Restrepo et al., 2018). In swine, mesothelial cells covering the ovarian bursa are cuboidal and  
281 biosynthetically activated, which suggests that these mesothelial cells may produce large amounts of surfactants  
282 and regulate immunomodulation, fluid balance, lubrication, and protection (Yániz et al., 2007). We report that the  
283 epithelium of the LOB in mice is partially lined with such cuboidal mesothelial cells. Hence, the LOB might have  
284 the unique function to regulate intrabursal immune balance and thereby promote healthy reproductive processes  
285 in the ovary. Previous studies reported that autoimmune disease in female MRL/lpr mice is severely exacerbated  
286 at 6 months of age, and the severe inflammation due to the infiltration of immune cells affects the ovaries and

287 oviduct (Otani et al., 2015; Hosotani et al., 2018). In this study, the lymphoma-like infiltration of inflammatory  
288 cells, including B cells, T cells, and macrophages was observed in the mesosalpinx folding the isthmus in MRL/lpr  
289 mice at 6 months of age. However, this was not observed in the LOB, thereby suggesting a higher ability of the  
290 LOB to regulate immune conditions compared to typical mesosalpinx. Further investigations will be needed to  
291 confirm these hypotheses.

292 Notably, the MRL/lpr at 6 months of age with severe autoimmune conditions lose their ovulated oocytes into  
293 the coeloma, which is neither the ovarian bursa nor the oviductal lumen (Hosotani et al., 2018). The diameter of  
294 mouse oocytes is approximately 80  $\mu\text{m}$  (Xiao et al., 2015). Based on our findings that the area of FOB in mice  
295 was approximately 0.04 to 0.12  $\text{cm}^2$ , and given smooth muscle contraction in the LOB might change the area of  
296 the FOB, we hypothesise that the oocytes released from the ovaries into the ovarian bursa can escape into the  
297 peritoneal cavity through the FOB in mice with autoimmune issues. In MRL/lpr mice in the severe disease stage,  
298 some of which possessed larger than average FOB relative to other strains and individuals, the altered morphology  
299 and function of the FOB might be one of the causes for the dysfunction of oocyte pick-up, in addition to the  
300 inflammation and abnormal morphology in the oviductal infundibulum (Hosotani et al., 2018).

301 The chronic inflammatory reactions triggered by persistent infections, autoimmune reactions, allergic  
302 responses, and tissue injury result in fibrosis (Wynn, 2008). In addition organs such as lungs, heart, liver, kidney,  
303 intestine, and skin (Wynn, 2008), the peritoneum is also pathologically affected by chronic inflammation and  
304 fibrosis (Wang et al., 2016). Even in MRL/lpr mice at 6 months of age which have severe inflammation in the  
305 mesosalpinx, there were only a small number of immune cells infiltrating the LOB. Nonetheless, the inflammation

306 enhances fibrosis in the LOB in MRL/lpr mice at 6 months of age, compared to 3 months of age, and MRL/+ at  
307 both ages. Once initiated, fibrogenesis in the intestine is no longer dependent on the presence of inflammation,  
308 suggesting that the fibrosis is self-propagating (Johnson et al., 2012). Although inflammation is prerequisite for  
309 the initiation of fibrosis, the severity of inflammation during fibrogenesis does not correlate with the degree of  
310 collagen deposition (Hünerwadel et al., 2018). Therefore, we consider that the severe and chronic systemic  
311 immune abnormalities, which deteriorate in the later life of MRL/lpr mice, alter the hormonal environment post-  
312 ovulation and immunological microenvironment, including cytokines in female reproductive organs. This leads  
313 to the deposition of thick collagen fibers in MRL/lpr mice at 6 months of age. The ultrastructure of the surface of  
314 the LOB revealed highly complicated LOB in MRL/lpr mice, which corresponds with the histological observation  
315 of thick collagen deposition underneath the mesothelium. Although it is unclear whether fibrosis affects the  
316 function of the LOB and morphology of the FOB, considering that B6 mice also possess the fibrotic LOB at both  
317 3 and 6 months of age, the deposition of collagen fibers in MRL/lpr mice in the severe disease stage might make  
318 the LOB stiffer than at younger ages. This can occur as organs increase their extracellular matrix, which contains  
319 fibrillar collagens (Wells, 2013), thus resulting in a stiffer LOB. Repeated ovulation induces an acute pro-  
320 inflammatory environment on the ovarian surface and oviductal fimbria, increasing ovarian cancer risk (Trabert  
321 et al., 2020). Although the ovulation rate of C57BL/6 and MRL strain mice is similar (about 10 oocytes per estrus)  
322 (Hosotani et al., 2019), the impaired clearance of intrabursal fluid containing inflammatory substances derived  
323 from ovarian follicles would cause pathology not only of the ovaries but also of the oviducts. The stiffer LOB  
324 perhaps affects the ovarian pathology due to impaired bursal fluid regulation in mice.

325 In conclusion, the ovarian bursa of mouse is connected to the peritoneal cavity, which is a characteristic similar  
326 to those of other mammals, such as ruminants and horses. We have also reported the physiological function of the  
327 LOB with a thick, smooth muscle layer in the maintenance of the fluid microenvironment and immune condition  
328 around the ovary. Further studies on the function of the FOB will provide a novel reproductive theory on  
329 maintenance of healthy ovulation and oocyte-pick-up by the oviductal infundibulum by regulating intraovarian  
330 bursal homeostasis.

### 332 **Acknowledgements**

333 This work was supported in part by JSPS KAKENHI (No. JP18J22313, 19K23708) and Rakuno Gakuen  
334 University Research Fund (No. 2020-01) (M. Hosotani). The research described in this paper was presented in  
335 part at the 162th Japanese Association of Veterinary Anatomists, 10-12 September 2019 in Ibaraki.

### 337 **Author contributions**

338 M. Hosotani designed and performed experiments, as well as analyzed the data. T. Namba performed the sampling  
339 of mice. O. Ichii, T. Nakamura, Y.H.A. Elewa and Y. Kon designed and reviewed the experiments. M. Hosotani,  
340 O. Ichii and Y. Kon wrote the manuscript. M. I. Rashedul, T. Watanabe, H. Ueda reviewed the manuscript. All  
341 authors approved the final manuscript.

### 343 **Conflicts of Interest**

344 The authors declare no conflicts of interest.

345

346 **Data availability statement**

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

348 request.

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454

455 **Figure legends**

456 **Figure 1. The stereomicroscopical morphology of the foramen of the ovarian bursa in mice.**

457 Arrows show the positions of the foramen of the ovarian bursa. The squares surrounded by dashed lines are  
458 magnified in images on the right side. The line drawings in the insets imitate the shape of the foramen of the  
459 ovarian bursa. Asterisk shows the infundibulum enveloped in the ovarian bursa of MRL/lpr mice at 6 months of  
460 age.

461 L: left, R: right, O: ovary, T: oviduct, U: uterus, †: ovarian cyst. B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr:  
462 MRL/MpJ-Fas<sup>lpr/lpr</sup>.

463

464 **Figure 2. Leakage of India ink from the ovarian bursa to extrabursa through the foramen of the ovarian**  
465 **bursa in mice.**

466 Arrows indicate the points where the India ink leaked (i.e., foramen of the ovarian bursa).

467 O: ovary, T: oviduct, U: uterus. B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-Fas<sup>lpr/lpr</sup>.

468

469 **Figure 3. The ultrastructure of the foramen of ovarian bursa in mice.**

470 Arrowheads indicate the spherical dome like mesothelial cells lining the LOB.

471 T: oviduct, U: uterus, FOB: the foramen of ovarian bursa, LOB: the ligament of ovarian bursa. B6: C57BL/6N,

472 MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-Fas<sup>lpr/lpr</sup>.

473

474 **Figure 4. 3D reconstruction of the female reproductive tract and the size measurement of the foramen of**  
475 **ovarian bursa in mice.**

476 (A) The mesothelium composing the female reproductive tract is colored in red. The foramen of ovarian bursa is  
477 colored in black and indicated by black arrowheads. The dashed yellow lines indicate the inside of the ovarian  
478 bursa, while the yellow arrows indicate the outside of the ovarian bursa. LOB: ligament of ovarian bursa, U:  
479 uterus.

480 (B) The peritoneal side area of the foramen of the ovarian bursa is measured.  $n = 4$  per group. There is no  
481 significant strain-related difference in the same age examined by the Kruskal-Wallis test followed by Scheffé's  
482 method and no significant differences between 3 and 6 months of age in the same strain examined by the Mann-  
483 Whitney  $U$ -test. Data is presented as the mean  $\pm$  SEM.

484 B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-Fas<sup>lpr/lpr</sup>.

485  
486 **Figure 5. The histology of the foramen and ligament of ovarian bursa in mice.**

487 The hematoxylin and eosin staining. The squares surrounded by black dashed lines are magnified in the images  
488 on the right side. The blue lines indicate the boundary of the ovarian bursa. Arrows connecting the intrabursal and  
489 peritoneal sides are passing through the foramen of the ovarian bursa, and the centers are indicated by asterisks.

490 Arrowheads indicate the cuboidal mesothelial cells lining the epithelium of the foramen of ovarian bursa. A:  
491 ampulla, B: ovarian bursa, In: infundibulum, Is: isthmus, O: ovary, Out: ostium uterinum tubae, U: uterus. B6:  
492 C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-Fas<sup>lpr/lpr</sup>.

493

494 **Figure 6. The distribution of smooth muscle cells and collagen fibers in the ligament of ovarian bursa in**  
495 **mice.**

496 The squares surrounded by black dashed lines are magnified in the insets. Asterisks: the foramen of ovarian bursa,  
497 Arrows: thick collagen fibers in the ligament of ovarian bursa,  $\alpha$ SMA: alpha smooth muscle actin,  
498 immunohistochemistry, MT: masson's trichrome staining. B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr:  
499 MRL/MpJ-Fas<sup>lpr/lpr</sup>.

500

501 **Figure 7. The immunohistochemistry of immune cells in the mesosalpinx suspending the oviductal isthmus**  
502 **in mice.**

503 Black circles surround the area of severe infiltration of immune cells. The squares surrounded by the black dashed  
504 lines are magnified in the insets, showing the area of significant infiltration of the immune cells. Arrows indicate  
505 the distribution of the Foxp3 positive cells. Is: isthmus. B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-  
506 Fas<sup>lpr/lpr</sup>.

507

508 **Figure 8. The immunohistochemistry of immune cells in the ligament of ovarian bursa in mice.**

509 Arrows indicate the distribution of immune cells in the connective tissue of the ligament of ovarian bursa.

510 Asterisks: the foramen of ovarian bursa. B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-Fas<sup>lpr/lpr</sup>.

511

512 **Figure 9. The anatomical schema of the foramen and ligament of ovarian bursa.**

513 The mesovarium and mesosalpinx enclose the peritoneal cavity, which is called the ovarian bursa, and the large  
514 part of ovary. The mesosalpinx connecting the oviductal ampulla or isthmus and the cranial part of uterus has the  
515 slit-like opening of the ovarian bursa to the peritoneal cavity, which is called the foramen of ovarian bursa. The  
516 ligament of ovarian bursa, which is the part of the mesosalpinx connecting the oviduct and uterus, surrounds the  
517 foramen of ovarian bursa. The ligament consists of a thick smooth muscle layer. The squares surrounded by  
518 dashed lines are magnified in the schema on the right side.

519  
520 **Supplementary Movie S1. Leakage of India ink from the ovarian bursa to extrabursa through the foramen**  
521 **of the ovarian bursa in C57BL/6N mouse at 3 months of age.**

522  
523 **Supplementary Figure S2. The ratio of spleen weight to body weight in mice.**

524 The strain-related difference in the same age is examined by the Kruskal-Wallis test followed by Scheffé's method.

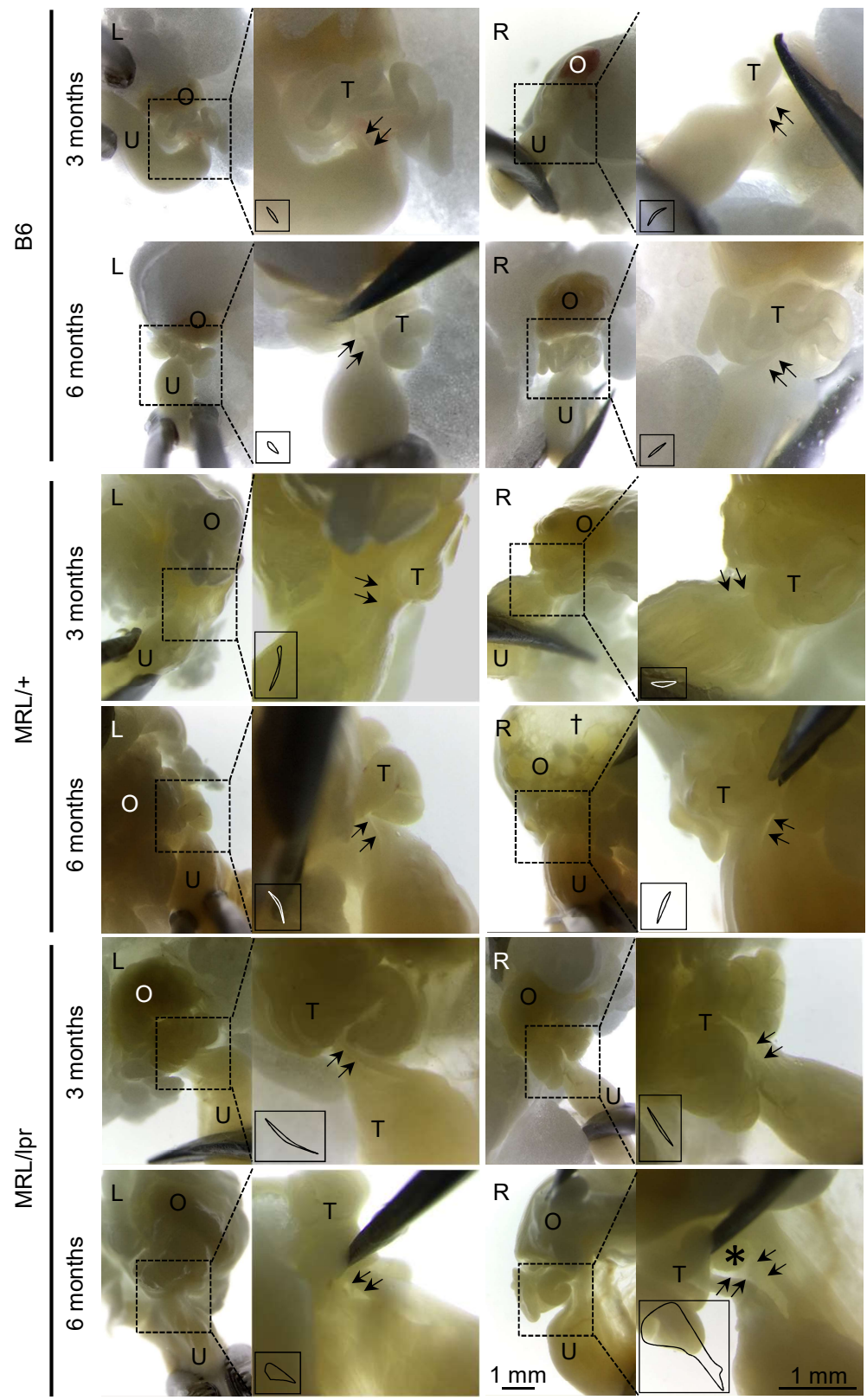
525 The differences between 3 and 6 months of age in the same strain is examined by the Mann-Whitney U-test. n =

526 5, 6 and 6 in B6, MRL/+ and MRL/lpr mice at 3 months of age and n = 4, 5 and 6 in B6, MRL/+ and MRL/lpr

527 mice at 6 months of age. Data is presented as the mean  $\pm$  SEM. **\*\*P < 0.01**

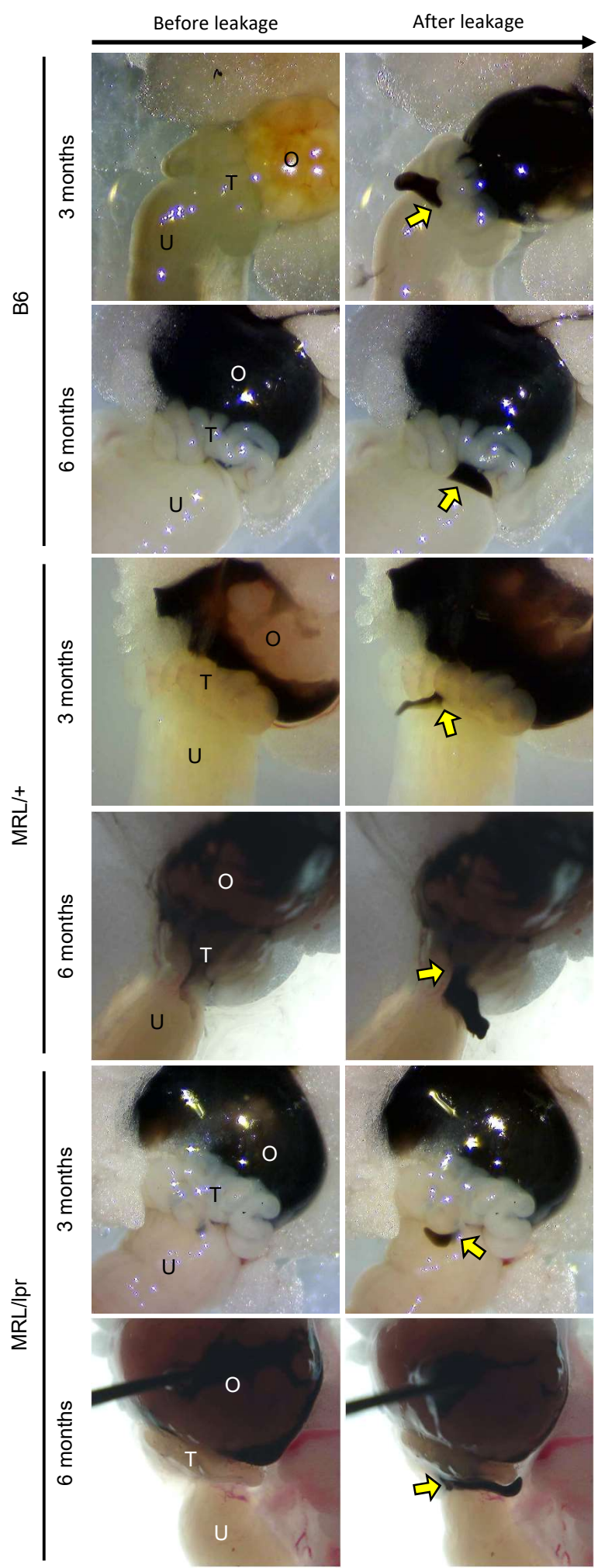
528 B6: C57BL/6N, MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-Fas<sup>lpr/lpr</sup>.

Figure 1

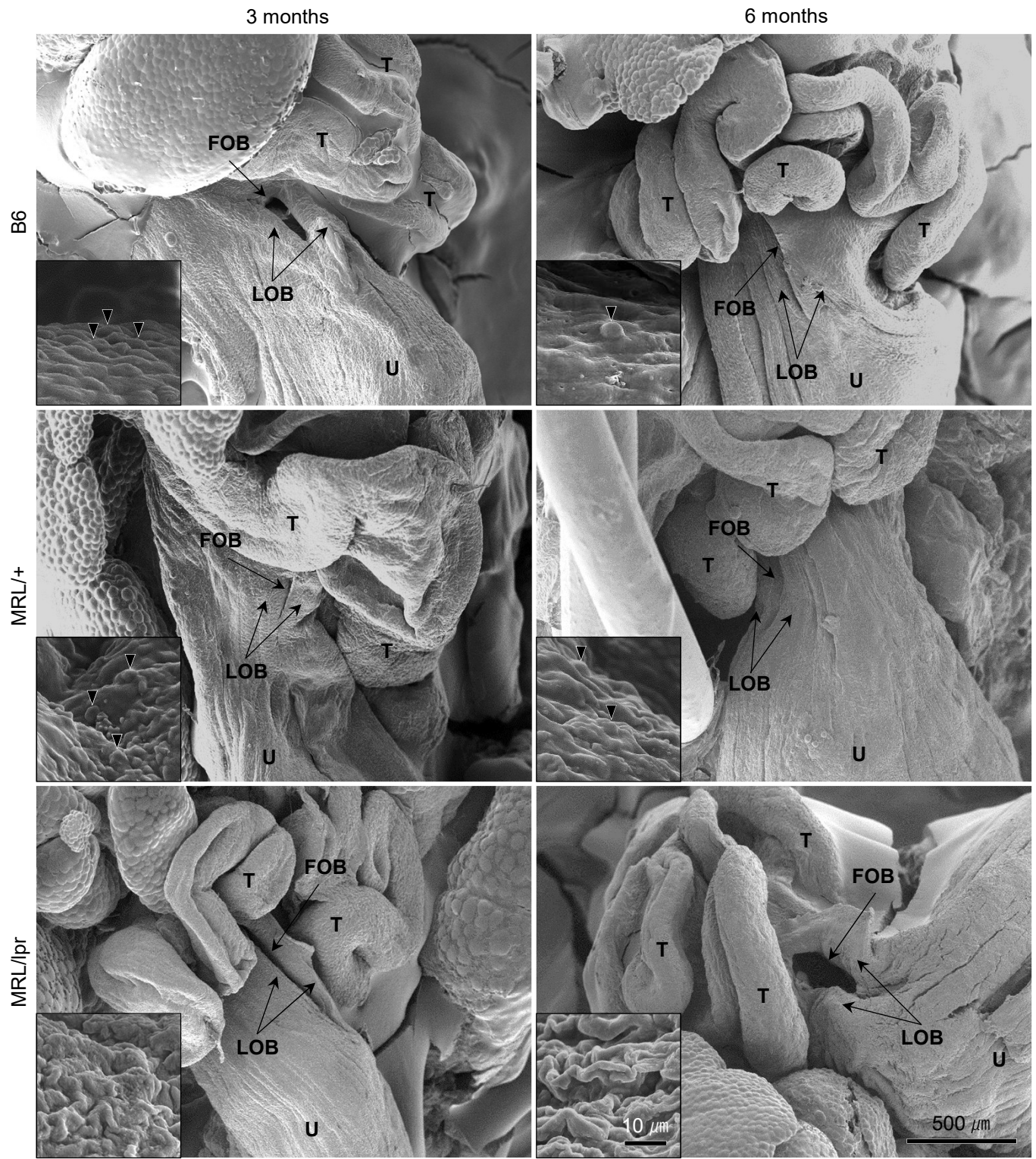




**Figure 2**



**Figure 3**



**Figure 4**

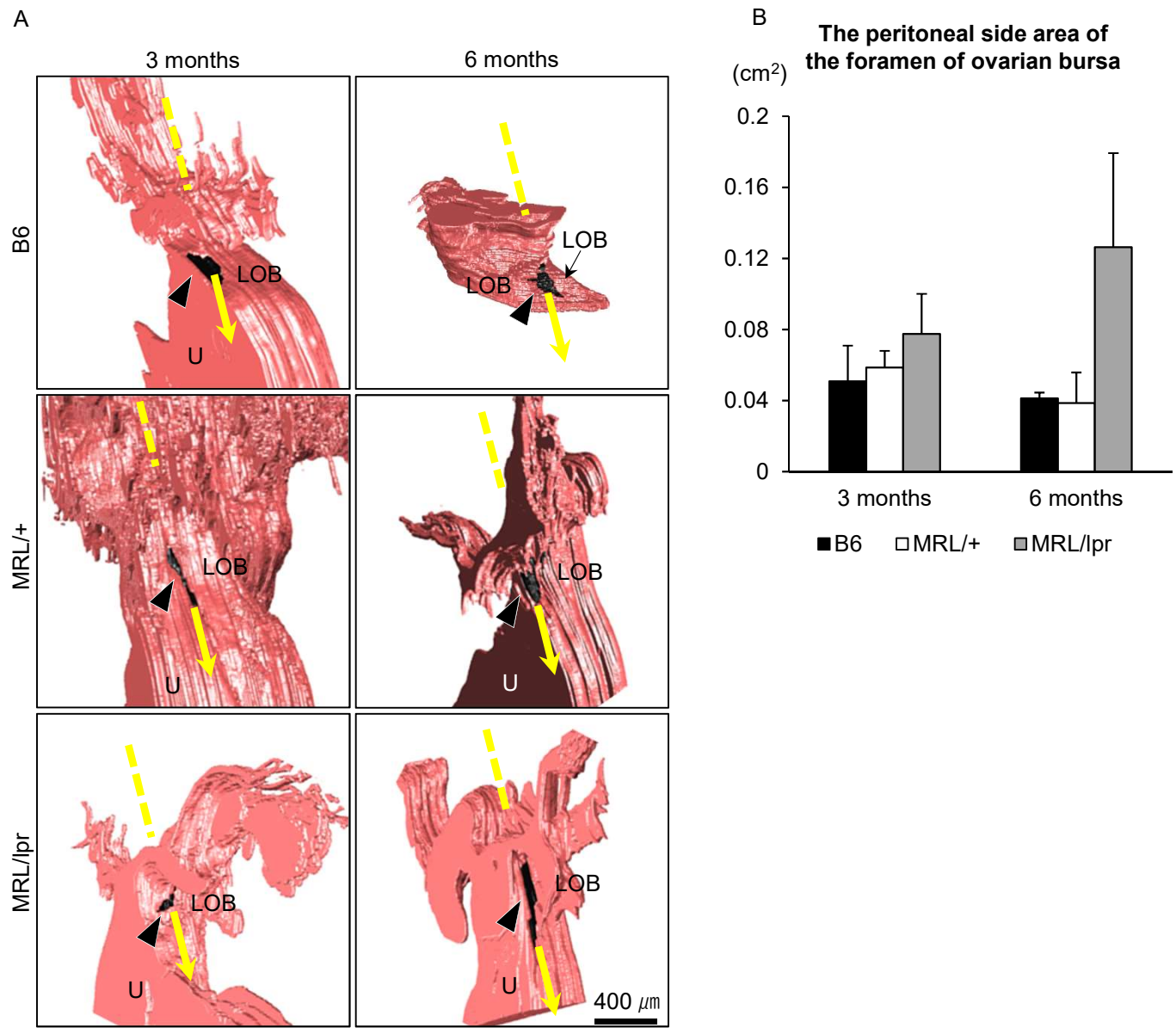
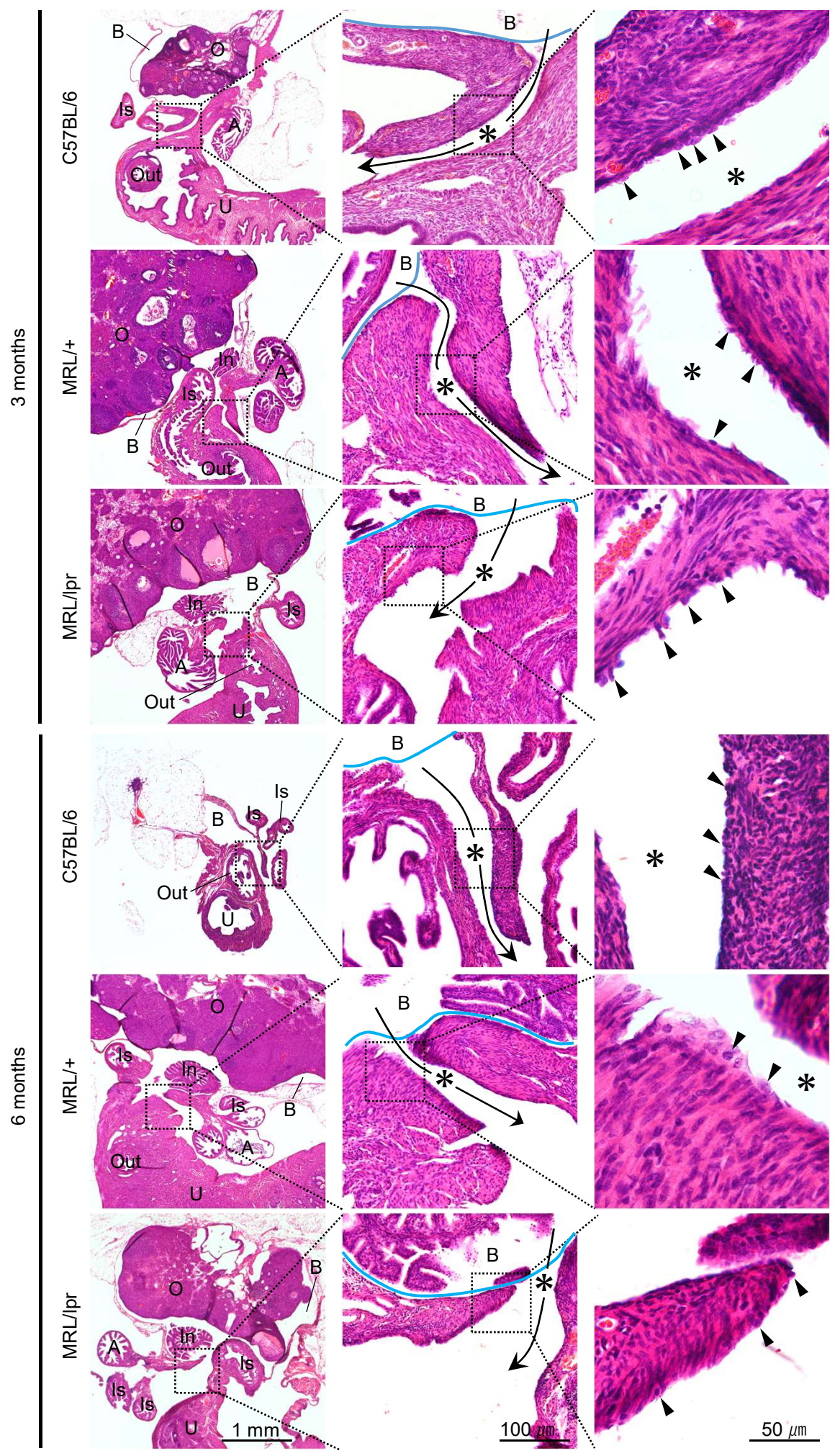




Figure 5





**Figure 6**

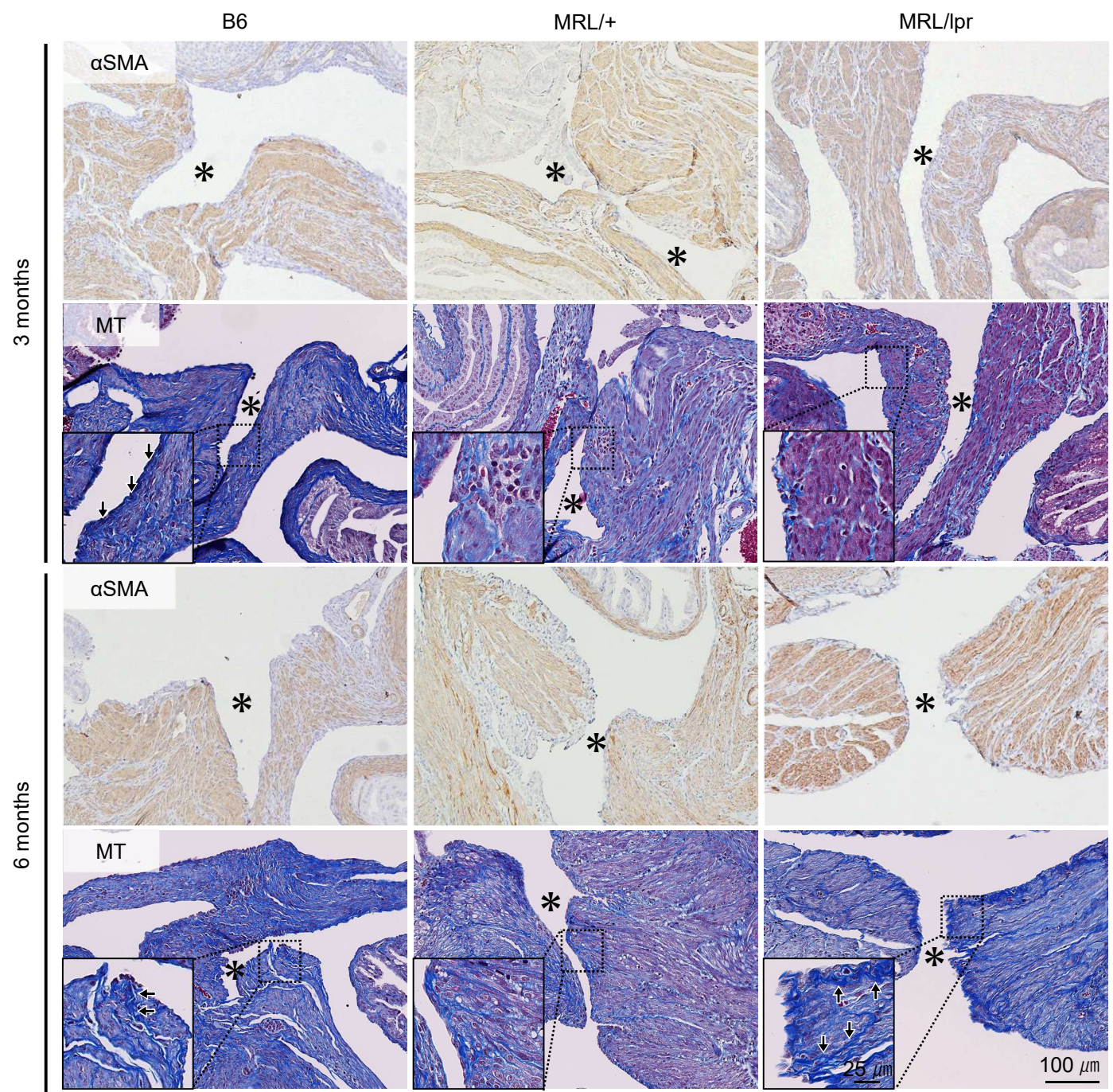
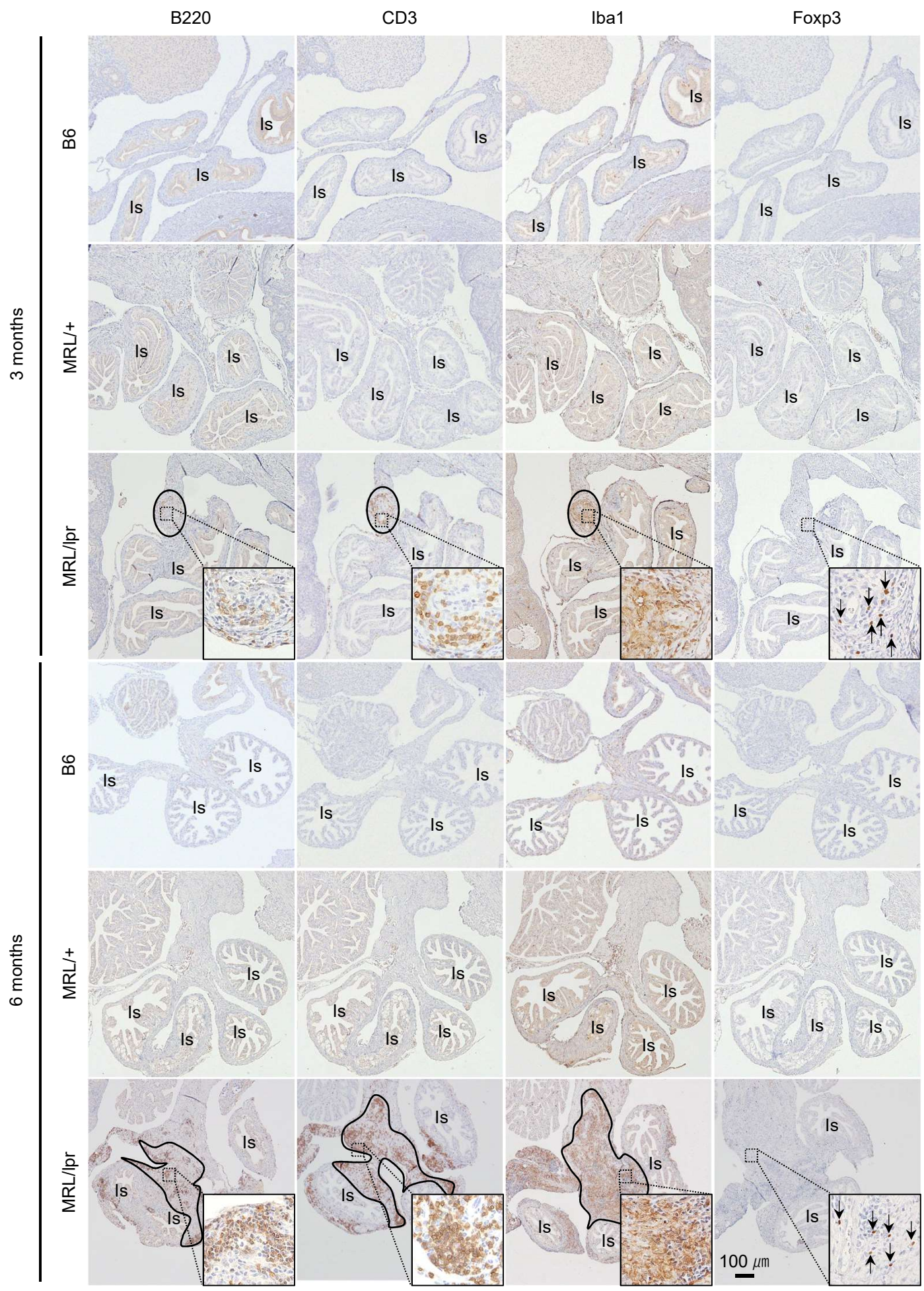


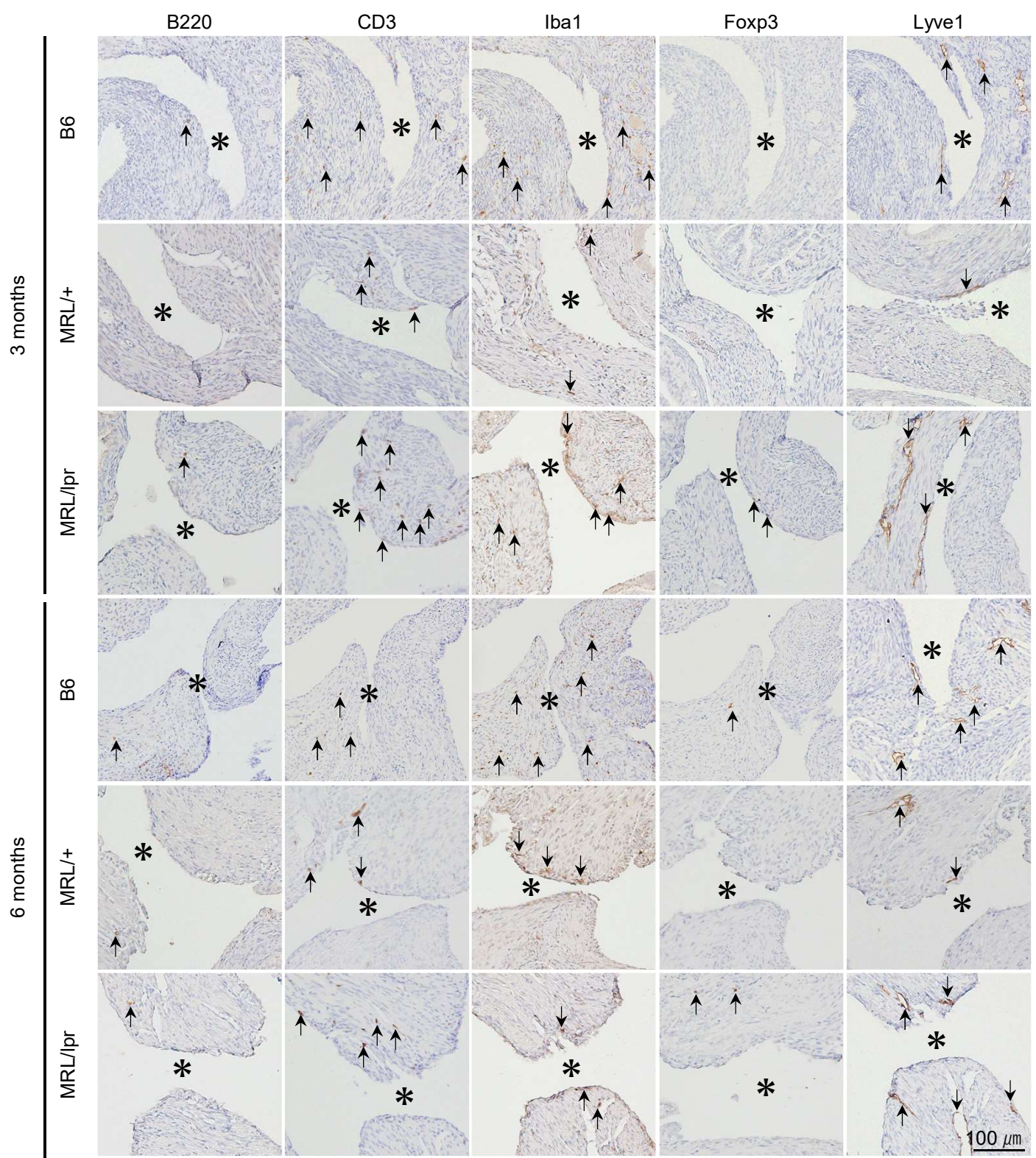


Figure 7





**Figure 8**



**Figure 9**

**Left side**

