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Original Article

Immunohistochemical identification of epithelial cell types in the isthmus of bovine oviduct: Comparison with the ampulla

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Abstract. The oviductal epithelium consists of ciliated and non-ciliated cells, and their numbers vary depending on the segment of the oviduct and stage of the estrous cycle. Compared with the ampulla, fewer cyclic changes in the number of the two types of cells occur in the isthmus. Recently, we have reported that the epithelium in the ampullary oviduct is composed of many types of cells during different translational/transcriptional states, and their numbers change during the estrous cycle. However, detailed information regarding the epithelial cell subtypes lining the isthmic oviductal epithelium has not yet been reported. In this study, we aimed to identify the epithelial subtypes in the isthmus of the oviduct using immunohistochemistry. Some similarities and differences were observed between the ampulla and isthmus. As observed in the ampulla, epithelial cells of the isthmus expressed either FOXJ1 (ciliogenesis marker) or PAX8 (non-ciliated cell marker). The estrous cycle affected the number of Ki67$^+$ cells but not that of ciliated cells. A relatively high rate of Ki67$^+$ cells (60%) was observed at 1–4 days after the ovulation. Interestingly, unlike the ampulla, Ki67$^+$/FOXJ1$^+$ cells (12.6 ± 1.1%) were discovered in the isthmus. Double staining for Ki67 with FOXJ1, PAX8, or Centrin-1 (a centriole marker) revealed that Centrin-1 was localized on the apical surface of some Ki67$^+$/FOXJ1$^+$ cells. In conclusion, some epithelial cell subtypes exist in the isthmus of the oviduct and isthmus-specific cell subtypes have been identified. These region-specific cells may provide functional and morphological differences between the ampulla and isthmus of the oviduct.
Key words: Characterization, Ciliated cells, Cow, Isthmic oviductal epithelium, Proliferation

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The oviduct is a tubular organ that links the ovary to the uterus, and it plays an essential role in successful pregnancy by providing an optimal microenvironment for fertilization and development of the embryo [1, 2]. The oviduct can be divided into the following three different regions: the fimbriae, ampulla, and isthmus [3]; their functions and structure differ depending on the segment of the oviduct. The fimbriae, which are present at the distal entrance of the oviduct, capture the oocyte after ovulation. The ampulla is the site where fertilization occurs, whereas the isthmus is a crucial region for early embryonic development and also acts as a sperm reservoir [4, 5]. The specific functions of each region of the oviduct can be attributed to two types of oviductal epithelial cells, namely ciliated and non-ciliated cells [6]. Ciliated cells have multiple cilia on their apical surfaces, and their movement promotes the transport of the zygote, oocyte, and early embryo [3]. Non-ciliated cells produce oviductal fluid, which supports early embryonic development [7]. The proportions of these cells differ among the oviductal regions [8]. In the ampulla, the epithelium contains a large number of ciliated cells. The isthmic epithelium contains a large number of non-ciliated cells and has a thicker layer of smooth muscle than that in the ampulla [9]. In cattle, ciliated cells are dominant during the follicular phase, whereas non-ciliated cells are abundant during the luteal phase [8].

In our previous study, we proposed that oviductal non-ciliated cells give rise to ciliated cells, which contributes to the changes in the proportions of ciliated and non-ciliated cells in the bovine oviduct [10]. Recently, we have reported that in terms of immunological classification, the epithelium in the ampullary oviduct is composed of many types of cells at different translational/transcriptional states, and their numbers change during the estrous cycle [11]. These changes in the oviductal epithelium appear to respond to the ovarian steroid hormones, viz. estradiol-17β and progesterone, as the oviduct is constantly exposed to these hormones.

Compared with the ampulla, there are fewer cyclic changes in the number of ciliated and non-ciliated cells in the isthmus of the oviduct during the estrous cycle [8, 12, 13], even though the isthmus of the oviduct is also affected by ovarian steroid hormones. However, the underlying mechanisms that cause regional differences during the estrous cycle have not yet been elucidated. Several studies have shown that the cellular response to ovarian steroid hormones or factors produced by the oviduct differs between the ampulla and isthmus [14, 15]. Moreover, the gene expression levels of sex steroid receptors and proteins secreted from the cells also differ among the regions of the oviduct [16, 17]. These findings raise the possibility that the epithelial cell subtypes differ between the ampulla and isthmus in the oviduct, although detailed information regarding the

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epithelial cell subtypes lining the isthmic oviductal epithelium has never been reported.

Therefore, the aim of the present study was to clarify epithelial cell subtypes in the isthmus of the oviduct.

Materials and Methods

Collection of bovine oviduct tissues

Bovine oviducts were collected at a local abattoir within 10–20 min after exsanguination. The isthmic parts of the oviductal tissues (the first 3 cm proximal to the utero-tubal junction [5]) were collected from cows at four different stages of the estrous cycle (stage I, days 1–4; stage II, days 5–10; stage III, days 11–17; and stage IV, days 18–20). The stages of the estrous cycle were determined based on macroscopic observations of the ovary and uterus [18–20]. The isthmic regions of the oviduct ipsilateral to the corpus luteum or the dominant follicle were fixed in 4% paraformaldehyde and then embedded in paraffin.

Immunohistochemistry and cell counting

Immunohistochemical analyses were conducted as previously described [10, 11]. The bovine oviductal sections of the isthmus (n = 6 per stage) were sliced to a thickness of 4 µm, microwaved in 0.1 M Tris-EDTA buffer (pH 9.0) for 15 min at 600 W to prepare the antigen, incubated in 5% bovine serum albumin-phosphate buffered saline (PBS) for 1 h at 18–25ºC to block non-specific binding, and then incubated with the relevant primary antibody overnight at 4ºC in a hydrated chamber. The following primary antibodies were used: mouse anti-acetylated-α-tubulin (1:500; T6793, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-FOXJ1 (1:100; HPA005714, Sigma-Aldrich), mouse anti-PAX8 (1:100; GTX101583, GeneTex, Los Angeles, CA, USA), mouse anti-Ki67 (1:200; M7240, Dako Cytomation, Glostrup, Denmark), and rabbit anti-Centrin-1 (1:100; GTX114316, GeneTex). The sections were washed thrice in PBS for 5 min and incubated with goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (1:500; A11008, Life Technologies, Carlsbad, CA, USA), goat anti-mouse IgG antibody conjugated with Alexa Fluor 594 (1:500; ab150116, Abcam, Cambridge, UK), donkey anti-rabbit IgG antibody conjugated with Alexa Fluor 568 (1:500; ab175693, Abcam), or goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:500; A11005, Life Technologies) for 60 min at 18–25ºC. Alexa Fluor 488 was used for green staining and Alexa Fluor 568 was used for red staining. The sections used as negative controls were incubated with the same concentration of mouse (ab37355, Abcam) or rabbit IgG (ab37415, Abcam) primary antibodies, followed by the incubation with secondary antibodies (isotype control). Finally, the sections were covered with ProLong Gold Antifade Reagent with DAPI (P36935, Life Technologies). The sections were mounted on an Olympus FSX100 microscope (Olympus, Tokyo, Japan) and observed under a 200X or higher objective. Cell counting was performed as previously described [11]. Briefly, the total number of positive and negative epithelial cells located on the 1 mm basement membrane was determined in three sections per oviduct in each immunohistochemical analysis.

Co-localization of FOXJ1, Ki67, and Centrin-1

In this experiment, co-localization of FOXJ1, Ki67, and Centrin-1 in the isthmus of the oviduct was investigated. Since anti-FOXJ1 and anti-Centrin-1 antibodies were produced in the same host (rabbit) and it was not possible to conduct triple staining for FOXJ1, Ki67, and Centrin-1, their co-localization was visualized on serial sections. One section was incubated with both anti-FOXJ1 and anti-Ki67 antibodies, and its consecutive section was incubated with anti-Centrin-1 antibody overnight at 4ºC, washed three times with PBS, and incubated with secondary antibodies for 60 min at 18–25ºC, followed by treatment with ProLong Gold Antifade Reagent with DAPI.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.03 (GraphPad Software, La Jolla, CA, USA). All experimental data are shown as the mean ± standard error of the mean (SEM). To check whether the data were parametric or non-parametric, they were tested for normal distribution and homogeneity of variance using the Shapiro–Wilk and Bartlett's tests. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used for the analysis of parametric data. The Kruskal–Wallis and Bonferroni tests were used for the analysis non-parametric data. P values less than 0.05 were considered to be statistically significant.

Results

Changes in the number of ciliated and non-ciliated cells during the estrous cycle

To clarify the changes in the number of ciliated and non-ciliated cells during the estrous cycle, we examined the number of cells that were positive or negative for acetylated-α-tubulin (cilia marker). Acetylated-α-tubulin$^+$ cells were observed in the isthmic oviductal epithelium (Figs. 1A, B). The number of acetylated-α-tubulin$^+$ cells (ciliated cells) and acetylated-α-tubulin$^-$ cells (non-ciliated cells) did not change significantly during the estrous cycle (Figs. 1C, D).

Changes in the number of FOXJ1$^+$ and PAX8$^+$ cells during the estrous cycle

Both FOXJ1 and PAX8 were detected in epithelial cells of the isthmus throughout the estrous cycle. Double immunostaining for FOXJ1 and PAX8 revealed that the epithelial cells in the isthmus expressed either FOXJ1 or PAX8 (Figs. 2A–C). The number of FOXJ1$^+$ and PAX8$^+$ cells did not change significantly during the estrous cycle (Figs. 2E, F). In the isthmus of the oviduct, 67.2 ± 1.0% (n = 6) of the total number of epithelial cells were positive for PAX8.

Changes in the number of Ki67$^+$ cells during the estrous cycle and co-localization of Ki67 and FOXJ1 or PAX8

To elucidate the cyclic changes in the number of proliferating cells during the estrous cycle and the types of cells that proliferate, we investigated the changes in the number of Ki67 (proliferation marker)-positive cells in the isthmus of the oviduct and performed double -staining for Ki67 and FOXJ1 or PAX8.

In the isthmus, Ki67$^+$ cells were observed throughout the estrous cycle. The number of Ki67$^+$ cells was the highest in stage I (Fig. 3A). In contrast, the total number of epithelial cells did not change significantly during the estrous cycle (Fig. 3B). Most of the Ki67$^+$ cells expressed PAX8 (Figs. 3C–E, 87.4 ± 1.1%), while 12.6 ± 1.1% of the Ki67$^+$ cells (n = 6) expressed FOXJ1 at stage I (Figs. 3G–I).

Co-localization of Centrin-1 and Ki67 or PAX8 in the isthmus of the oviduct

We next investigated whether the ciliated cells could proliferate. In this experiment, Centrin-1, which is located at the base of the cilia [21, 22], was used as a marker for ciliated cells because some non-ciliated cells were covered by cilia extruded from the ciliated cells and would have otherwise been misidentified as ciliated cells.

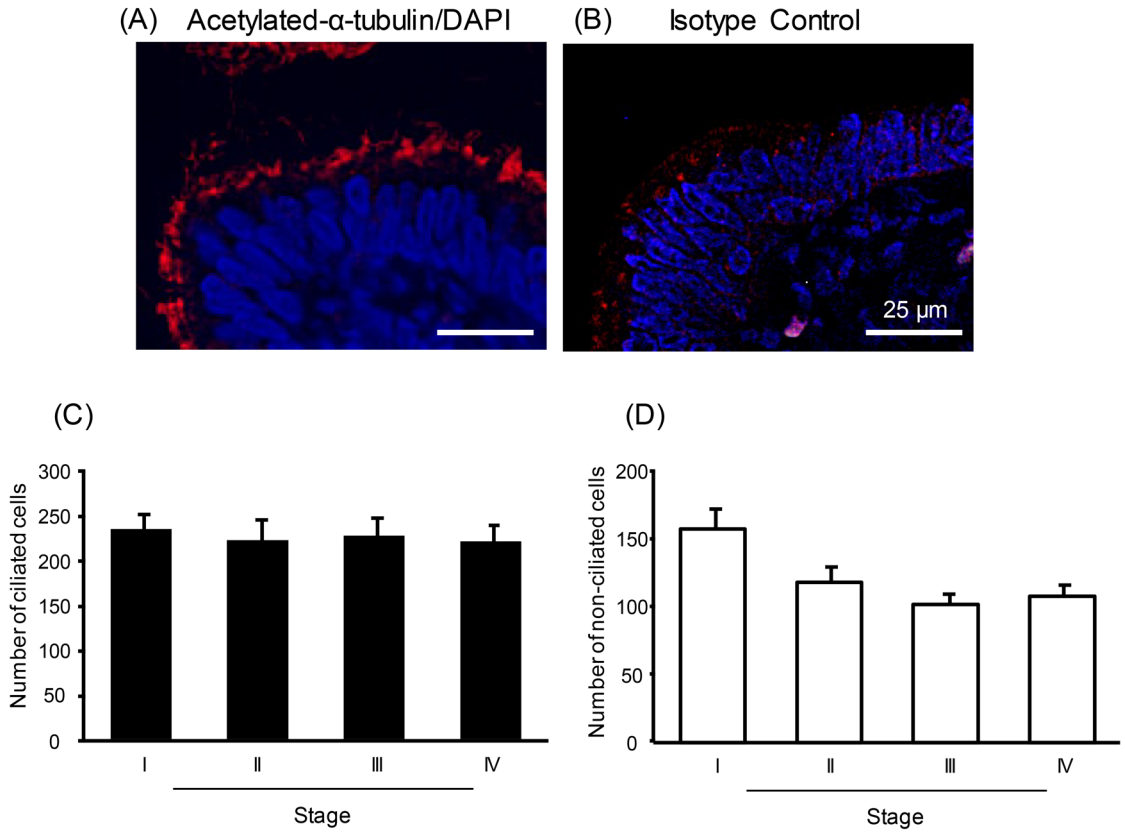

Fig. 1. Changes in the number of cells positive or negative for acetylated-α-tubulin in the isthmus of the bovine oviduct. (A) Immunohistochemical localization of cells showing positive signal for acetylated-α-tubulin in the isthmus of the oviduct, and (B) isotype control. Scale bars represent 25 μm. Changes in the numbers of (mean ± SEM, n = 6) (C) ciliated and (D) non-ciliated cells in the isthmus of the bovine oviduct during the estrous cycle.

Some Ki67$^+$ cells were negative for Centrin-1, while other Ki67$^+$ cells were positive for Centrin-1 (Figs. 4A–C). In stage I, 11.3 ± 1.7% of Ki67$^+$ cells (n = 6) were positive for Centrin-1 in the isthmus of the oviduct. We confirmed that all Centrin-1$^+$ cells were negative for PAX8 (Figs. 4D–F) and positive for acetylated-α-tubulin (Figs. 4 G–I).

Co-localization of Centrin-1, Ki67, and FOXJ1 in the isthmus of the oviduct

To identify Centrin-1$^+$/Ki67$^+$/FOXJ1$^+$ cells in the isthmus of the oviduct, immunohistochemical staining was performed on consecutive serial sections. Because the oviductal cells are far larger than 4 μm, consecutive serial sections still contain the same cells.

As shown in Fig. 5, Centrin-1 was found to be localized on the apical surface of some of the FOXJ1$^+$/Ki67$^+$ cells (Fig. 5).

Discussion

The proportions of ciliated and non-ciliated cells change during the estrous cycle [8]. In the previous study, we investigated the mechanisms underlying these changes in the ampulla of the bovine oviduct [10, 11]. To clarify the mechanisms of the cyclic morphological changes in the isthmus of the oviduct, epithelial cell subtypes and their numbers were immunohistochemically examined in the present study; Some similarities and differences were observed when compared to the ampulla.

FOXJ1 is a transcription factor that induces the formation of cilia by regulating the expression of ciliogenesis-related factors [23, 24]. PAX8 is known as a non-ciliated cell marker in the oviduct [25, 26]. As shown in Figs. 2A–C, FOXJ1 and PAX8 double-positive cells were not observed in the isthmus. This finding was consistent with our previous results showing that oviductal epithelial cells in the ampulla express either FOXJ1 or PAX8 [11].

Acetylated-α-tubulin is widely used as a marker for ciliated cells [27, 28]. In the ampulla of the oviduct, the number of acetylated-α-tubulin$^+$ cells and FOXJ1$^+$ cells changes during the estrous cycle [11]. In the isthmus, as shown in Figs. 1 and 2, the number of acetylated-α-tubulin$^+$ cells and FOXJ1$^+$ cells did not change significantly during the estrous cycle. These results are in agreement with a previous study reporting that few cyclic morphological changes are observed in the isthmus of the bovine oviduct [8]. Prior to the appearance of cilia, an increased FOXJ1 expression is observed in the airway and oviductal epithelium [11, 23, 29]. As the number of FOXJ1$^+$ cells in the isthmus was not affected by the estrous cycle, the number of acetylated-α-tubulin$^+$ cells might have remained unchanged during the estrous cycle. It is known that the ampulla and isthmus are two anatomically and functionally different regions of the oviduct [6, 16, 30, 31]. The epithelium of the ampulla has a higher number of folds than that of the isthmus, and the transport of the oocyte and embryo is promoted by the oviductal fluid flow produced by ciliary beating and smooth muscle motility [9]; the isthmus has a more muscular wall than the ampulla and transports embryos to the uterus by peristaltic contractions of smooth muscle [1]. Considering that ciliary beating during the transport of the embryo in the isthmus is less important than that in the ampulla, it is assumed that a dramatic

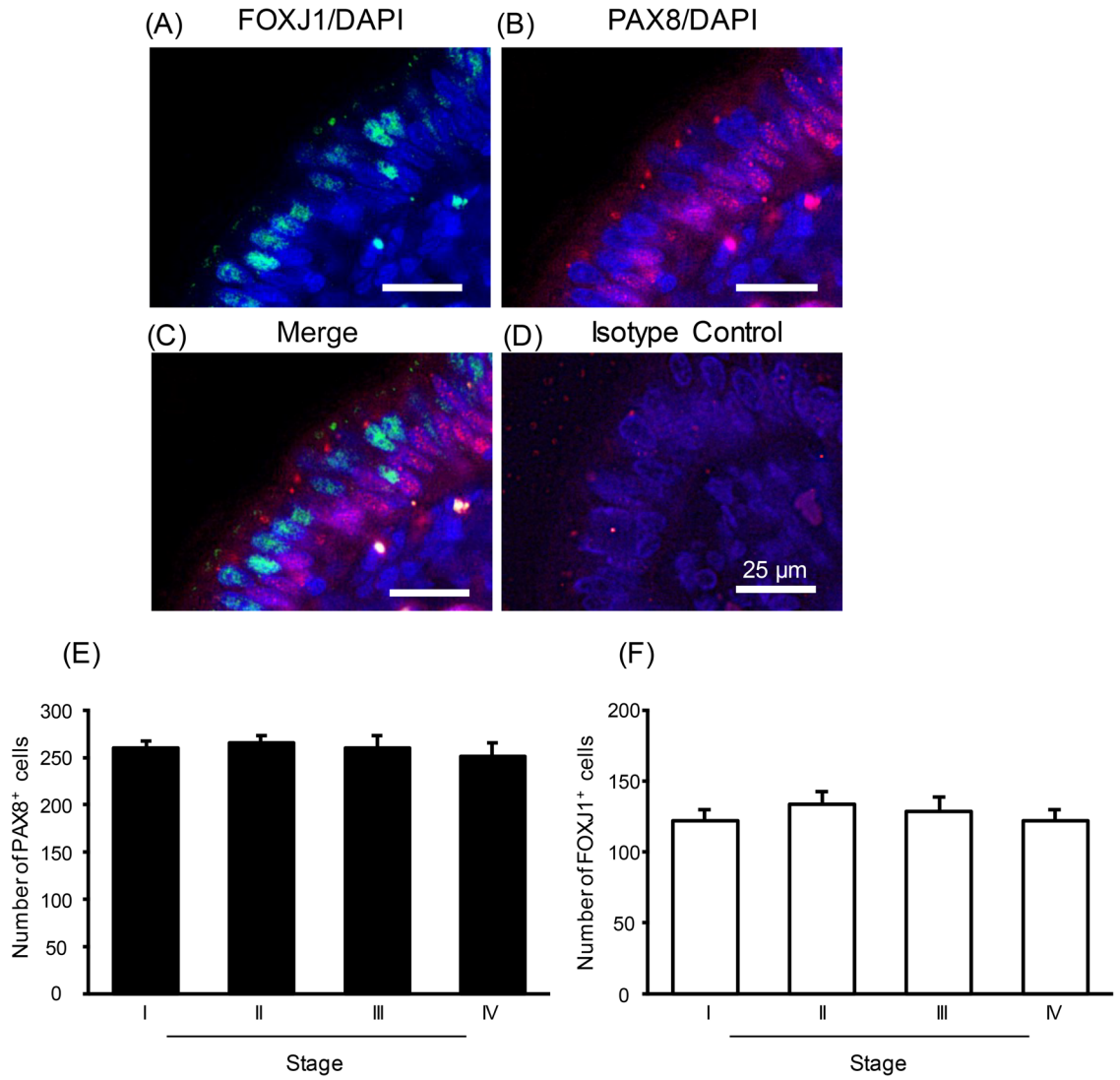

Fig. 2. Changes in the number of FOXJ1$^+$ and PAX8$^+$ cells in the isthmus of the bovine oviduct during the estrous cycle (mean ± SEM, n = 6). (A–C) Immunohistochemical detection of PAX8 (red) and FOXJ1 (green) in the isthmus at stage I. The cell nuclei were counterstained with DAPI (blue). (D) Image depicting the isotype control. The scale bars represent 25 μm. Changes in the numbers of (E) PAX8$^+$ cells and (F) FOXJ1$^+$ cells in the isthmus during the estrous cycle.

increase in the number of ciliated cells around the time of ovulation is not necessary in the isthmus.

Although an estrous cycle-dependent change in the number of Ki67$^+$ cells was observed in both the ampulla and isthmus of the oviduct, the extent of this change varied according to the segment of the oviduct. As reported in our previous study, the highest number of Ki67$^+$ cells in the ampulla (approximately 10% of the total epithelial cells) is observed in stage IV [11]. In contrast, in the isthmus, the number of Ki67$^+$ cells reached its maximum at stage I and comprised 67.3 ± 4.3% of the total epithelial cells (Fig. 3A). These results suggest that cell proliferation in the oviduct is regulated in a specific manner in each segment of the oviduct. The concentration of estradiol-17β (E2) in the oviductal tissue is highest in stage I [32]. When ovulation occurs, follicular fluid containing a high concentration of E2 along with several growth factors enters the oviduct [33–37]. Growth factors are known to influence the proliferation of a variety of cells [38, 39]. Taken together, proliferation of the epithelial cells in the ampulla might be regulated by E2. On the other hand, it is possible that the proliferative activity in isthmic epithelial cells is controlled not only by E2 but also by growth factors in the follicular fluid. Moreover, transport through the oviduct takes 3 to 4 days after ovulation, and each segment of the oviduct performs different functions within this limited timeframe [40]. The ampulla provides an environment for egg transport and fertilization, while the isthmus supports the development of the early embryos. Thus, the optimal environment differs depending on the particular region of the oviduct. To prepare an optimal environment for fertilization and early development, the proliferation of epithelial cells may be temporally and spatially controlled during the estrous cycle. These differences between the ampulla and isthmus might provide a time lag during the increase in Ki67$^+$ cells in the bovine oviduct. Interestingly, the total number of cells in the isthmus of the oviduct remained unchanged during the estrous cycle (Fig. 3B). We have previously reported that cleaved caspse-3 positive cells were observed in the isthmus oviductal epithelial cells [46], and that the number of these cells was the highest at stage I when the number of proliferating cells was increased. These data suggest

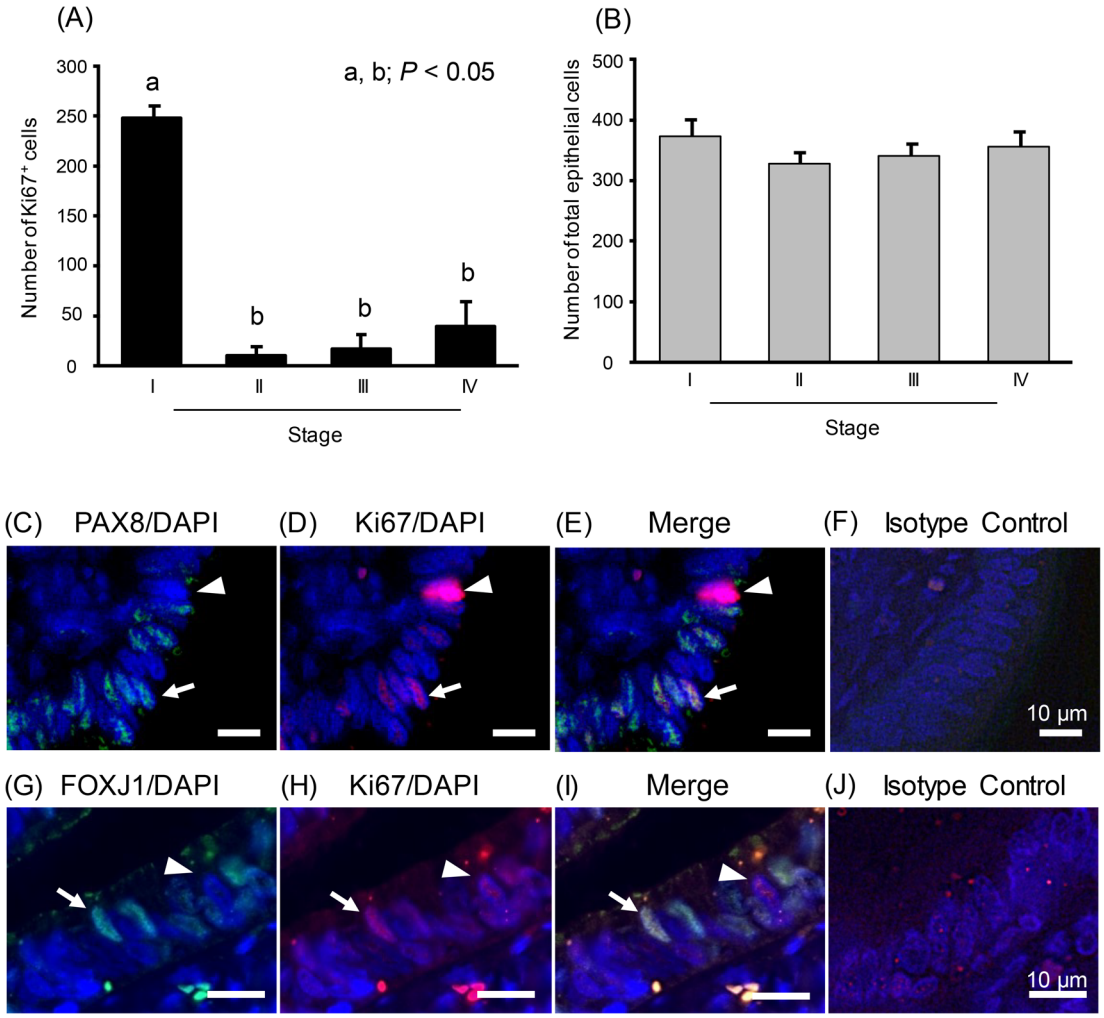

Fig. 3. Changes in the number of Ki67$^+$ cells in the isthmus of the bovine oviduct during the estrous cycle (mean ± SEM, n = 6). Changes in the number of (A) Ki67$^+$ cells and (B) total epithelial cells in the isthmus of the oviduct during the estrous cycle. Different superscript letters indicate significant differences (P < 0.05). (C–E) Immunohistochemical detection of Ki67 (red) and PAX8 (green) in the isthmus at stage I. Arrows indicate cells that were double-positive for Ki67 and PAX8, while arrowheads indicate cells that were positive for Ki67 and negative for PAX8. (G–I) Immunohistochemical detection of Ki67 (red) and PAX8 (green) in the isthmus at stage I. Arrows indicate cells that were double-positive for Ki67 and FOXJ1, while arrowheads indicate cells that were positive for Ki67 and negative for FOXJ1. Cell nuclei were counterstained with DAPI (blue). (F, J) Image depicting the isotype control. Scale bars represent 10 μm.

that the unwanted cells from the isthmic oviductal epithelium are removed by apoptosis.

The present study revealed that some epithelial cell subtypes found in the ampulla of the oviduct were also observed in the isthmus [11]. However, region-specific cells were identified in the isthmus. We discovered cells that were double-positive for Ki67 and FOXJ1, which were absent in the ampulla (Figs. 3G–I). Double staining for Ki67 and Centrin-1 revealed that some Ki67$^+$ cells co-expressed Centrin-1. Given that all epithelial cells were positive for either FOXJ1 or PAX8 (Fig. 2A-C) and that none of the PAX8$^+$ cells co-expressed Centrin-1 (Figs. 4E–G), Centrin-1$^+$/Ki67$^+$ cells might be positive for FOXJ1. As shown in Fig.5, FOXJ1$^+$/Ki67$^+$ cells co-expressed Centrin-1 in the isthmus of the oviduct. Moreover, we confirmed that all Centrin-1$^+$ cells were positive for acetylated-α-tubulin (Figs. 4I–K). In light of these results, isthmic ciliated cells may have the potential to proliferate. As in the bovine oviduct, acetylated-α-tubulin$^+$/Ki67$^+$ cells have been observed in the mouse oviduct as well [41]. In general, ciliated cells are considered terminally differentiated cells [42]. In contrast, some cell types, such as retinal pigment epithelial cells, have only one cilium on their surface in the G0 phase and can re-enter the cell cycle [43]. The disassembly of the primary cilium begins once these cells re-enter the cell cycle [44, 45]. The relationship between the cell cycle and disassembly of cilia in ciliated oviductal cells was not elucidated in this study. Further studies are required to address this issue.

In conclusion, epithelial cell subtypes are dependent on the segment of the oviduct, and their population is regulated in a region-specific manner. Isthmic oviductal epithelial cells may provide an optimal environment for fertilization, sperm storage, and early embryonic development through a region-specific proliferation mechanism.

Conflict of interests: The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the reported research.

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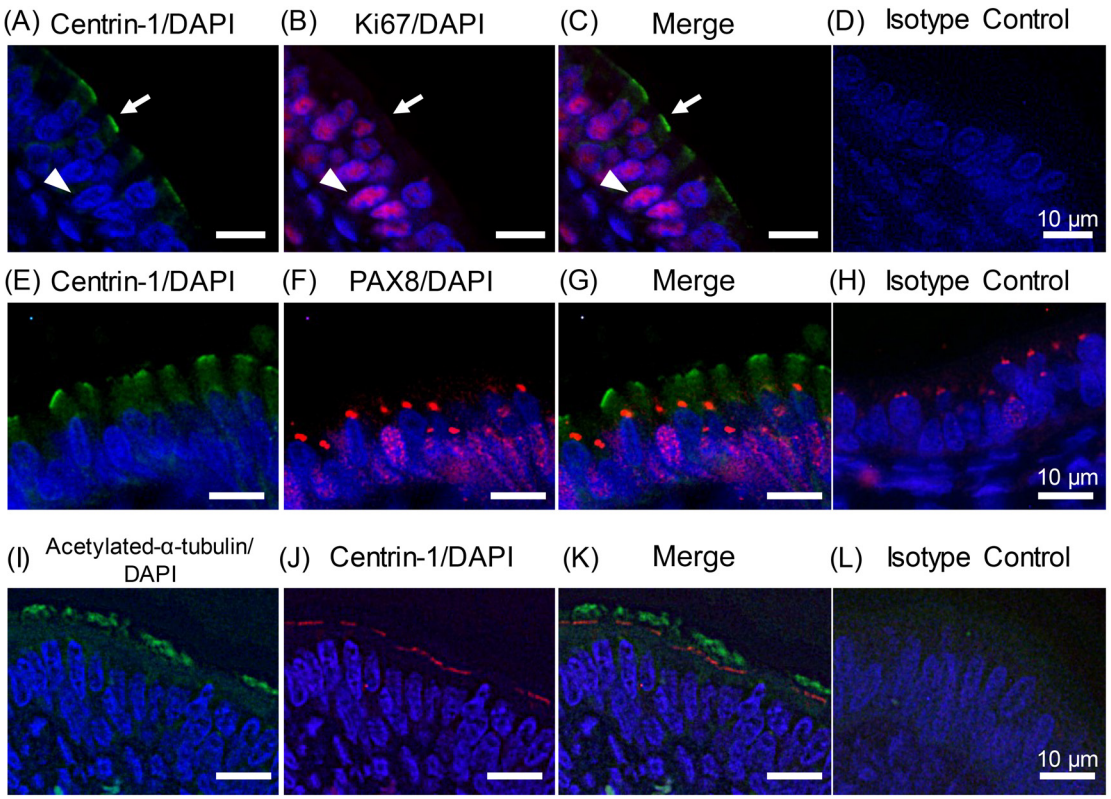

Fig. 4. Localization of Centrin-1, Ki67, PAX8, and acetylated-α-tubulin in the isthmus of the oviduct. (A–C) Immunohistochemical detection of cells showing positive signal for Centrin-1 (green) and Ki67 (red) at stage I. Arrows indicate cells that were double-positive for Centrin-1 and Ki67, while arrowheads indicate cells that were positive for Centrin-1 and negative for Ki67. (E–G) Immunohistochemical detection of cells showing positive signal for Centrin-1 (green) and PAX8 (red) at stage I. (I–K) Immunohistochemical detection of cells positive for acetylated-α-tubulin (green) and Centrin-1 (red) in the isthmus of the oviduct at stage I. Cell nuclei were counterstained with DAPI (blue). (D, H, L) Image depicting the isotype control. Scale bars represent 10 μm.

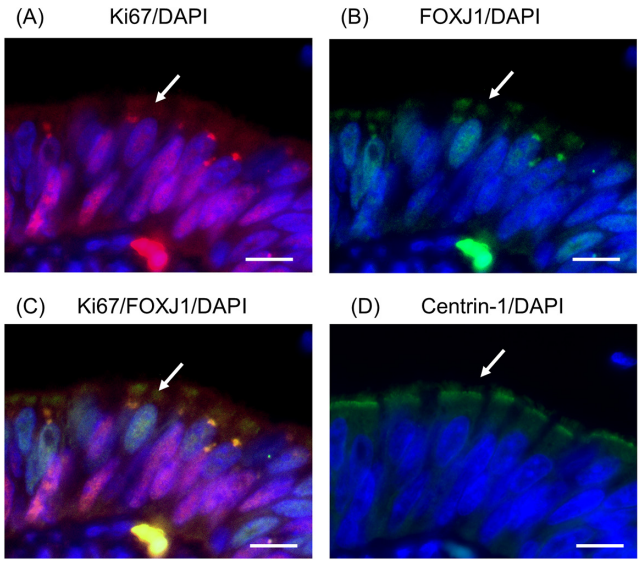

Fig. 5. Co-localization of FOXJ1, Centrin-1, and Ki67 in the isthmus of the oviduct. Double-immunostaining was performed on serial sections of the oviduct using anti-FOXJ1 and anti-Ki67 antibodies or anti-Centrin-1 antibody. (A–C) Immunohistochemical detection of cells positive for Ki67 (red) and FOXJ1 (green) in the isthmus of the oviduct at stage I. (D) Immunohistochemical detection of cells positive for Centrin-1 (green) in the isthmus of the oviduct at stage I. The same cells are indicated by the arrows. Cell nuclei were counterstained with DAPI (blue). Scale bars represent 10 μm.

for providing bovine oviducts.

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