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Anatomy

Localization of putative pituitary stem/progenitor cells in female dairy cattle

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ABSTRACT. Research on sex-determining region Y-box 2 (SOX2)-positive pituitary stem/progenitor cells, as a source of hormone-producing cells, is progressing rapidly in rodents. However, the stem/progenitor cells supplying hormone-producing cells that are essential for growth, reproduction, and lactation in bovines have not yet been identified. In this study, we characterized SOX2-positive cells in the pituitary gland of dairy cattle (Holstein heifers) after sexual maturity. Immunofluorescence analysis revealed that the localization pattern of SOX2-positive cells in the dairy cattle pituitary gland was similar to that observed in the rodent pituitary gland; the marginal cell layer (MCL), dense cell clusters, and single cells scattered in the parenchyma of the anterior lobe. Furthermore, most of the SOX2-positive cells were positive for the pituitary stem/progenitor cell niche markers E-cadherin and cytokeratin 8+18, which have been reported in rodents. In addition, in the MCL of the anterior lobe, there was a subpopulation of SOX2-positive cells positive for paired-related homeobox 1 and 2, whereas negative for S100β. Moreover, in the parenchyma of the anterior lobe, co-localization of SOX2 and pituitary hormones was infrequent. In summary, this study reveals the localization of putative pituitary stem/progenitor cells positive for SOX2 in dairy cattle. These results provide valuable information to support further investigation of cell supply in the dairy cattle pituitary gland.

KEY WORDS: dairy cattle, pituitary gland, sex-determining region Y-box 2, stem/progenitor cell

The pituitary gland is a master endocrine organ composed of three lobes (the anterior, intermediate, and posterior lobes). Among them, the anterior lobe comprises five types of hormone-producing cells (somatotrophs, thyrotrophs, gonadotrophs, mammotrophs, and corticotrophs), together with non-hormonal cells such as stem/progenitor cells, folliculo-stellate cells and fenestrated sinusoids (i.e., endothelial cells and pericytes), and plays important roles in physiological processes such as growth, metabolism, reproduction, lactation, and stress response, via the secretion of hormones. The number of hormone-producing cells is maintained by the proliferation and differentiation of pituitary stem/progenitor cells, the cell differentiation considered particularly important [7, 8, 18].

Stem/progenitor cells, which can divide frequently and regularly to replenish the exhausted or damaged cells by either natural course or injury throughout the whole life, and are known to maintain their stemness in the microenvironment, the so-called niche, in some organs (ex. the brain, intestine, and skin) [2]. However, different organs have different strategies to regulate their stem/progenitor cells. In the past 20 years, studies in mice and rats have reported that pituitary stem/progenitor cells in the anterior lobe are composed of subpopulations of sex-determining region Y-box 2 (SOX2)-positive cells [1, 3, 7, 9, 21], and able to differentiate into all types of hormone-producing cells. In the adult rodent pituitary gland, these SOX2-positive cells are located densely in the marginal cell layer (MCL), which faces the residual lumen of Rathke's pouch (Rathke's cleft), and in cell clusters scattered in the parenchyma of the anterior lobe [4, 14, 22]. Thus, the MCL (MCL-niche) and SOX2-positive dense cell clusters scattering in the parenchyma of the anterior lobe (parenchymal-niche) are postulated as niches [20]. Of note, immunofluorescence analysis also demonstrated that SOX2-positive cells exist in humans [8] and in the common marmoset [19], which has been increasingly employed as a non-human primate model. However, research on pituitary stem/progenitor cells in other mammals has hardly progressed.

Anterior pituitary hormones are closely related to processes such as growth, reproduction, and lactation. Therefore, in cattle, which are representative animals that supply humans with milk and meat worldwide, it is important to identify the pituitary stem/progenitor cells that supply hormone-producing cells and understand the mechanism of cell differentiation. Nagai *et al*. (2008)

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

Table 1. List of antibodies used for immunofluorescence analysis

Antigen	Species	Label	Cat. No	Identifier
Human SOX2 (1:400)	Goat	None	GT15098	Neuromics, Minneapolis, MN, USA
Cow Cytokeratin 8+18 (1:200)	Guinea pig	None	ab194130	Abcam, Cambridge, UK
Human PRRX2 (1:500)	Mouse	None	H00051450-A01	Abnova, Taipei, Taiwan
Human Ki67 (1:100)	Mouse	None	350501	BioLegend, San Diego, CA, USA
Human E-cadherin (1:100)	Rabbit	None	#3195	Cell Signaling Technology, Danvers, MA, USA
Cow S100 (1:500)	Rabbit	None	IS504	Dako, Carpinteria, CA, USA
Human POMC (1:800)	Rabbit	None	#23499	Cell Signaling Technology
Canine LHβ (1:2,000)	Rabbit	None	HAC-CN27-02-RBP97	Kindly provided by the Institute for Molecular and Cellular Regulation (IMCR), Gunma University, through the courtesy of Dr. K. Sato
Canine TSHβ (1:8,000)	Rabbit	None	HAC-CN29-02-RBP97	
Rat GH (1:2,000)	Rabbit	None	HAC-RT25-02-RBP85	
Rat PRL (1:10,000)	Rabbit	None	HAC-RT26-01-RBP85	

SOX2, sex-determining region Y-box 2; PRRX2, paired-related homeobox 2; POMC, proopiomelanocortin; LHβ, luteinizing hormone beta-subunit; TSHβ, thyroid-stimulating hormone beta-subunit; GH, growth hormone; PRL, prolactin.

reported that, in the bovine pituitary gland, interleukin 18-positive cells, which are localized to both the MCL and the parenchyma of the anterior lobe, express pituitary stem/progenitor cell markers such as *Oct4*, *Nanog*, *Nestin,* and *Pitx1* [12]. However, isolated interleukin 18-positive cells have been shown to be immune-negative for S100β [12]. At first, S100β is found as a marker for folliculo-stellate cells [15]. Furthermore, since the majority of S100β-positive cells in the adult rodent pituitary gland express SOX2, it has been proposed that S100β/SOX2 double-positive cells are pituitary stem/progenitor cells responsible for the supply of hormone-producing cells [1, 22]. That is, in the rodents, a subpopulation of S100β-positive cells is pituitary stem/progenitor cells. On the other hand, research on bovine pituitary stem/progenitor cells has barely progressed over the past 10 years.

Here, we analyzed the stem/progenitor cells in the pituitary gland of sexually matured female dairy cattle, focusing on SOX2-positive cells. Immunofluorescence analysis revealed that SOX2-positive cells are widely distributed throughout the pituitary gland, especially in the MCL and in clusters present in the parenchyma of the anterior lobe. In addition, most of SOX2-positive cells, which were located in the MCL and the parenchymal cell clusters, co-localized with E-cadherin and cytokeratin (CK) 8+18 known as the pituitary stem/progenitor cell niche marker in the rodent [4, 5]. Furthermore, co-localization of SOX2 with paired-related homeobox 1 and 2 (PRRX1+2) and a proliferation cell marker Ki67 was observed in the MCL and the parenchymal cell clusters of the anterior lobe, but no co-localization with S100β. Altogether, the results in the present study provide novel information about stem/progenitor cells in the bovine pituitary gland, which can serve as a source of hormone-producing cells.

MATERIALS AND METHODS

Animals

Pituitary glands (n=3) from one-year-old female dairy cattle (Holstein heifers) were kindly provided by Tottori Livestock Hygiene Service Center of Tottori Prefecture (Tottori, Japan).

Immunofluorescence analysis

The pituitary glands of female dairy cattle were immersed with 4% paraformaldehyde in 20 mM HEPES buffer (pH 7.5) immediately after dissection (within about 30 min after euthanasia) and fixed for 24 hr at 4°C, followed by immersion in 30% trehalose in 20 mM HEPES buffer for 48 hr at 4°C to cryoprotect the tissues. Glands were then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan) and frozen immediately, followed by preservation storage at −80°C before sectioning. Frozen sections (8-µm thick) were prepared from the coronal planes of the pituitaries. Immunofluorescence was performed as previously described [9, 14]; the primary antibodies listed in Table 1 were used. In brief, the sections were incubated with 10% (v/v) fetal bovine serum and 0.4% (v/v) Triton X-100 in HEPES buffer (blocking buffer) for 60 min at room temperature, followed by incubation with primary antibodies in blocking buffer at room temperature overnight. Afterward, the sections were incubated with the secondary antibodies Alexa Fluor 488-conjugated AffiniPure bovine anti-goat and Cy3-conjugated AffiniPure donkey anti-rabbit, -mouse, or -guinea pig IgGs (1:500; Jackson ImmunoResearch, West Grove, PA, USA). Sections were then mounted with VECTASHIELD Mounting Medium containing 4,6-diamidino-2-phenylindole (DAPI, Vector, Burlingame, CA, USA), and immunofluorescence was detected using a fluorescence microscope (BZ-9000/BZ-X810; Keyence, Osaka, Japan). The number of cells positive for SOX2 and hormone antibodies and the total number of cells (DAPI-staining cells) per area were counted, and the proportion of each cell type was calculated.

The absence of an observable nonspecific reaction was confirmed with normal rabbit, goat, mouse, or guinea pig sera. In addition, to examine the specificity of antibodies, immunofluorescence analysis was performed with each primary antibody preabsorbed with recombinant proteins [0.03 mg/ml SOX2, PRRX1, luteinizing hormone β-subunit (LHβ), and prolactin (PRL)] or proteins purified from the bovine pituitary gland [0.03 mg/ml growth hormone (GH, MP Biomedicals, Santa Ana, CA, USA) and thyroid-stimulating hormone β-subunit (TSHβ, Sigma-Aldrich, St. Louis, MO, USA)]. No immunoreactivity was found with

preabsorbed antibodies (Supplementary Fig. 1). However, the anti-PRRX2 antibody recognized bovine PRRX1, similar to previous findings in rats [17]. Therefore, the results of immunofluorescence analysis using the anti-PRRX2 antibody were described as PRRX1+2. On the other hand, antibodies against E-cadherin, CK8+18, Ki67, S100β, and proopiomelanocortin (POMC), which is a precursor of alpha-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), have been shown to be cross-reactive with cow as described in the data sheet.

Hematoxylin and eosin (H&E) staining

Frozen sections were stained with Mayer's hematoxylin solution for 4 min at room temperature and then rinsed in tap water until the water was colorless, followed by staining with 1.0% eosin Y solution for 2 min. Next, the section was treated stepwise with 70–100% ethanol and xylene, and then mounted with EUKITT mounting medium (O. Kindler, Freiburg, Germany). The image was detected using a light microscope (Eclipse Ts2-FL; Nikon Instech, Tokyo, Japan).

RESULTS

Localization of SOX2-positive cells in the pituitary glands of female dairy cattle

H&E-stained sections in the pituitary gland of female dairy cattle are shown in Fig. 1. A cell layer in contact with Rathke's cleft was observed on the anterior and intermediate lobe sides (Fig. 1B). There was a dense cell cluster in the parenchyma of the anterior lobe (Fig. 1C). In addition, there were several vacuolar structures with blood cells (i.e. blood vessels) in the parenchyma of the anterior lobe (Fig. 1D). Immunofluorescence analysis revealed that SOX2-positive cells were widely distributed throughout the pituitary glands of dairy cattle (Fig. 2A). Furthermore, SOX2-positive cells were localized densely in the area facing Rathke's cleft, the MCL (Fig. 2B). In the parenchyma of the anterior lobe, although most of the SOX2-positive cells were singly scattered, dense SOX2-positive cell clusters were also detected (Fig. 2C). In each individual, the proportion of SOX2-positive cells (SOX2-positive cells / total cells stained with DAPI / 0.24 mm^2 in each section) in the anterior lobe was 20.1 ± 1.3% (Subject 1), 21.3 ± 0.9% (Subject 2), and 19.4 ± 0.3% (Subject 3) (4 sections / each subject). These data showed no differences between individuals.

Co-localization of SOX2 and niche markers in the dairy cattle pituitary gland

To analyze whether SOX2-positive cells localize in the pituitary stem/progenitor cell niches (the MCL and parenchymal niches), we performed double-immunostaining using antibodies against SOX2 and E-cadherin or CK8+18, epithelial stem/cell niche markers [4, 5]. Interestingly, most of SOX2-positive cells located in the MCL were positive for E-cadherin and CK8+18 (Fig. 3A and 3C). In addition, dense SOX2-positive cell clusters in the parenchyma of the anterior lobe were also positive for both niche markers, but some cells were negative (Fig. 3B and 3D). These results show that SOX2-positive cells in the dairy cattle pituitary gland are localized in two niches defined in mammals such as rodents and humans.

Co-localization of SOX2 and other stem/progenitor cell or proliferative cell markers in the dairy cattle pituitary gland

To investigate the heterogeneity of SOX2-positive cells in the pituitary gland of female dairy cattle, we performed double-immunostaining for SOX2 and other pituitary stem/progenitor cell markers, S100β or PRRX1+2. No co-localization of SOX2 and S100β was observed in both the MCL and parenchyma of the anterior lobe (Fig. 4A and 4B). In the MCL of the anterior lobe, SOX2

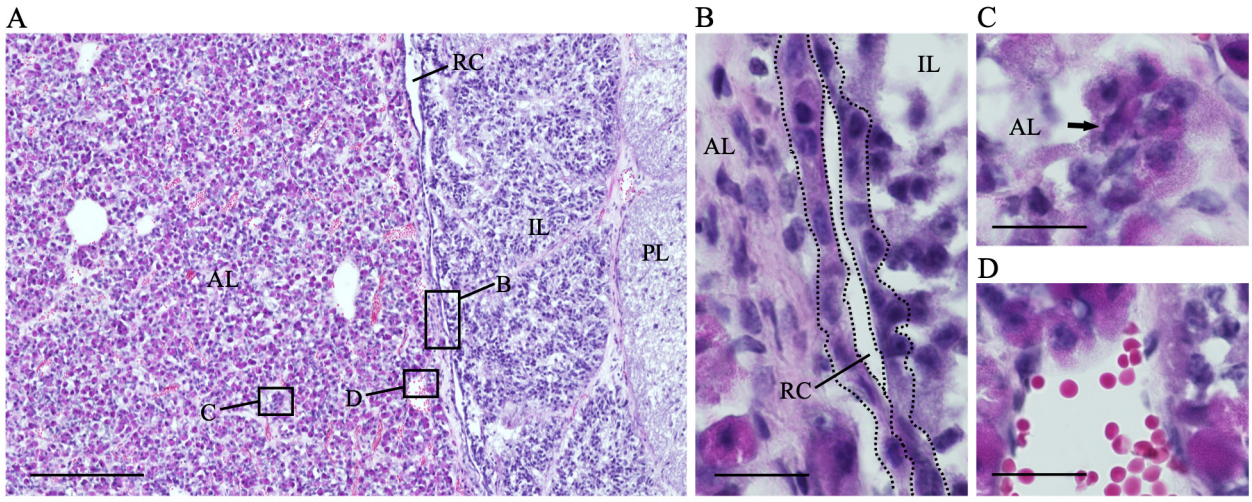

Fig. 1. Histological analysis in the dairy cattle pituitary gland. (A) Frozen sections of the pituitary glands were stained with hematoxylin and eosin, and photographed. The boxed areas are enlarged in B–D, respectively. The dotted lines refer to the marginal cell layer, and the arrow indicates the dense cell cluster. Scale bars=200 μm (A), 20 μm (B–D). AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe; RC, Rathke's cleft.

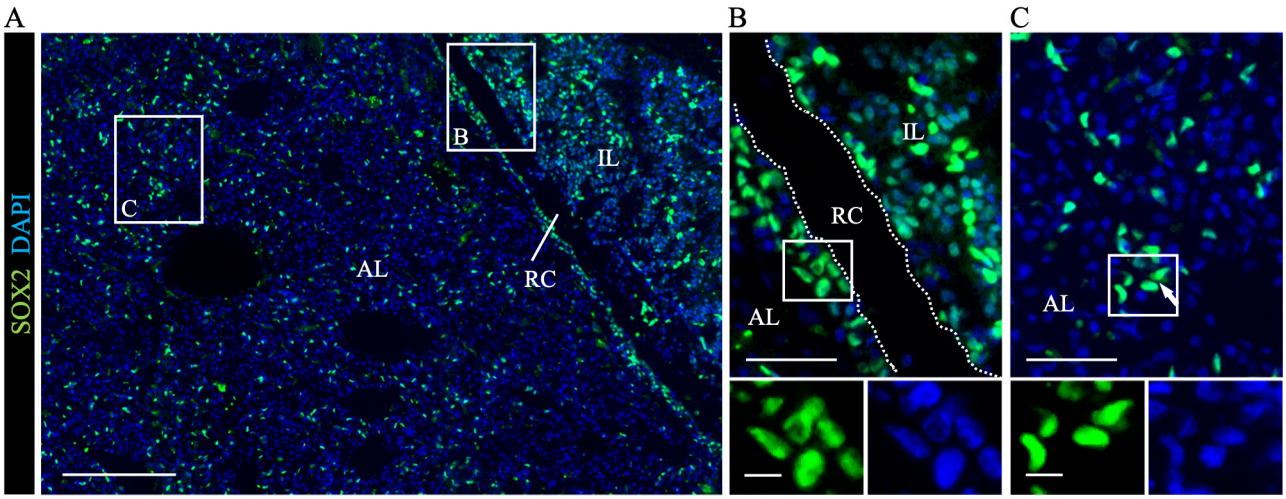

Fig. 2. Immunofluorescence analysis for sex-determining region Y-box 2 (SOX2) in the dairy cattle pituitary gland. (A) SOX2 immunofluorescence (green) was detected on the pituitary glands of dairy cattle. Images merged with DAPI signals (nuclei staining, blue) are shown. The boxed areas are enlarged in upper panels of B and C. Each enlarged image of SOX2 and DAPI in boxed areas is shown in lower panels. The dotted lines and arrow refer to the marginal cell layer and the dense SOX2-positive cell cluster, respectively. Scale bars=200 µm (A), 50 µm (upper panels in B, C), 10 µm (lower panels in B, C). AL, anterior lobe; IL, intermediate lobe; RC, Rathke's cleft.

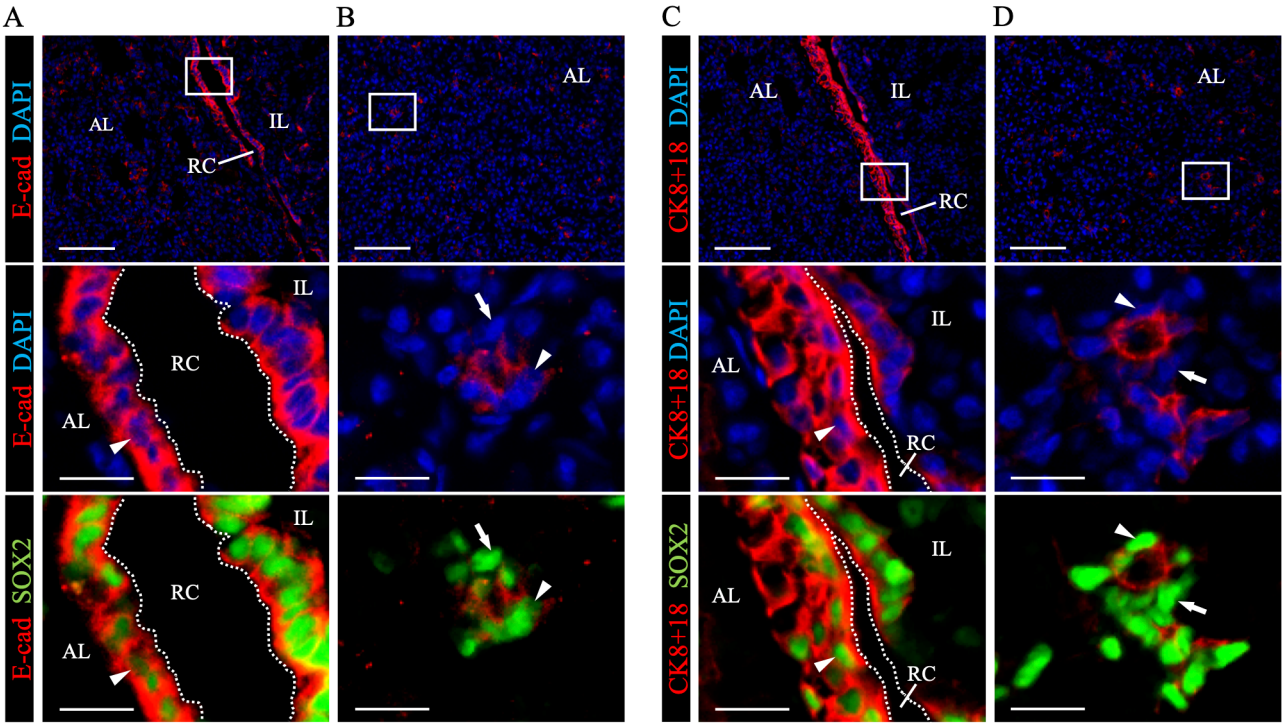

Fig. 3. Immunofluorescence analysis for sex-determining region Y-box 2 (SOX2) and stem/progenitor cell niche markers in the dairy cattle pituitary gland. (A–D) Double-immunostaining for SOX2 (green) and E-cadherin or cytokeratin (CK) 8+18 (red) on the marginal cell layer (the dotted lines) and the parenchyma of the anterior lobe in the pituitary glands of dairy cattle was performed, together with DAPI (nuclei staining, blue). Merged images are shown in upper panels, and the boxed areas are enlarged in middle and lower panels. Arrows and arrowheads show SOX2 single- and SOX2/E-cadherin (CK8+18) double-positive cells, respectively. Scale bars=100 µm (upper panels), 20 µm (middle and lower panels). AL, anterior lobe; IL, intermediate lobe; RC, Rathke's cleft.

mostly co-localized with PRRX1+2 at a high frequency, whereas SOX2-single positive cells were also detected (Fig. 4C). On the other hand, SOX2-positive cells in the parenchymal cell cluster of the anterior lobe co-localized with PRRX1+2, but PRRX1+2-single positive cells were also scattered in the parenchyma (Fig. 4D). To analyze whether SOX2-positive cells proliferate, we

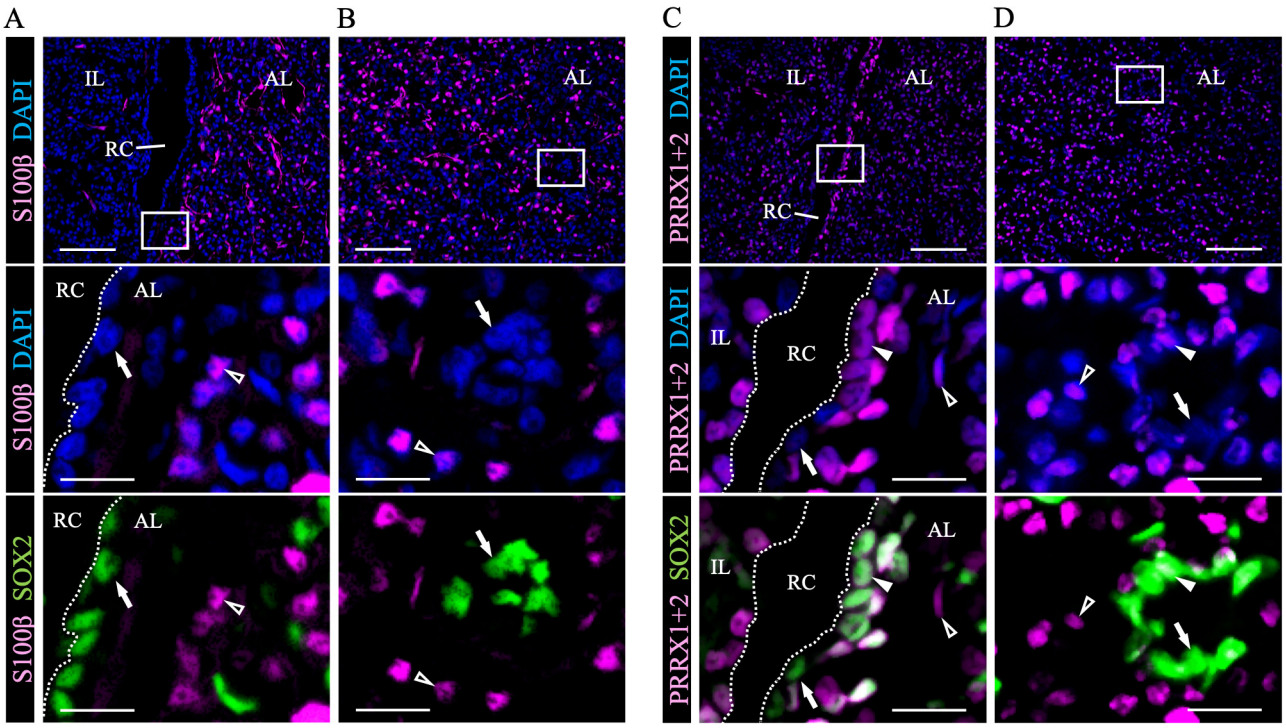

Fig. 4. Immunofluorescence analysis for sex-determining region Y-box 2 (SOX2) and other stem/progenitor cell markers in the dairy cattle pituitary gland. (A–D) Double-immunostaining for SOX2 (green) and S100β or PRRX1+2 (purple) on the marginal cell layer (the dotted lines) and the parenchyma of the anterior lobe in the pituitary glands of dairy cattle was performed, together with DAPI (nuclei staining, blue). Merged images are shown in upper panels, and the boxed areas are enlarged in middle and lower panels. Arrows, open arrowheads and closed arrowheads show SOX2 single-, S100β (PRRX1+2) single-, and SOX2/PRRX1+2 double-positive cells, respectively. Scale bars=100 µm (upper panels), 20 µm (middle and lower panels). AL, anterior lobe; IL, intermediate lobe; RC, Rathke's cleft.

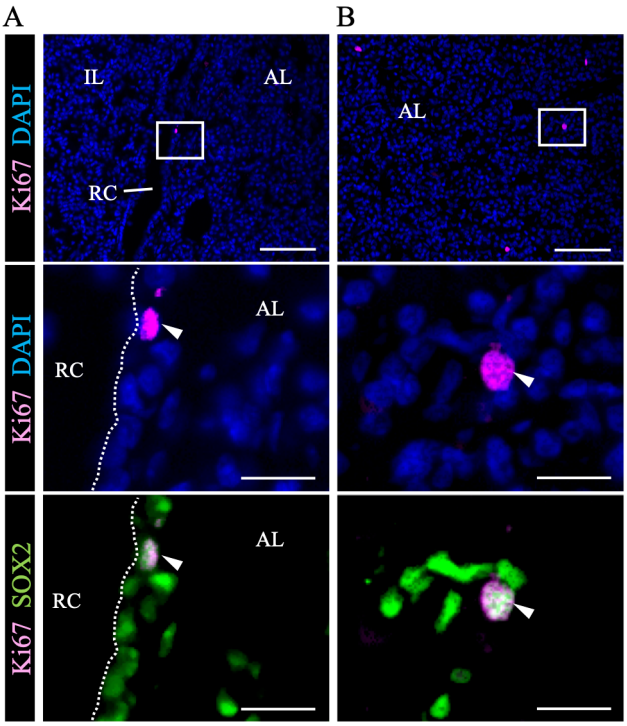

Fig. 5. Immunofluorescence analysis for sex-determining region Y-box 2 (SOX2) and the proliferative cell marker in the dairy cattle pituitary gland. (A, B) Double-immunostaining for SOX2 (green) and Ki67 (purple) on the marginal cell layer (the dotted lines) and the parenchyma of the anterior lobe in the pituitary glands of dairy cattle was performed, together with DAPI (nuclei staining, blue). Merged images are shown in upper panels, and the boxed areas are enlarged in middle and lower panels. Arrowheads show SOX2/Ki67 double-positive cells. Scale bars=100 µm (upper panels), 20 µm (middle and lower panels). AL, anterior lobe; IL, intermediate lobe; RC, Rathke's cleft.

performed double-immunostaining using antibodies against SOX2 and Ki67, a proliferative cell marker. As a result, although Ki67-positive cells were very rare in the MCL and the parenchyma of the anterior lobe, most of them were positive for SOX2 (Fig. 5A and 5B). These results indicate the heterogeneity and proliferative activity of SOX2-positive cells in the dairy cattle pituitary gland.

Co-localization of SOX2 and hormones in the dairy cattle pituitary gland

Finally, we performed immunofluorescence analysis to evaluate the co-localization of SOX2 and pituitary hormones (Fig. 6A). The hormone-positive rate among total cells (stained with DAPI) was 12.6 ± 1.1% (ACTH), 11.3 ± 1.4% (LHβ), 3.6 ± 1.5% (TSHβ), 29.5 ± 3.5% (GH), and 20.6 ± 3.5% (PRL) (Fig. 6B, n=4 in each hormone). Moreover, α-MSH-positive cells were detected in the intermediate lobe. Next, double-positive cells for SOX2 and each hormone were counted to examine whether SOX2 is present in a transitional state of terminal differentiation into hormone-producing cells. The coexistence of SOX2 was observed at a low frequency in all types of hormone-producing cells (Fig. 6C, ACTH: 0.92 ± 0.18%, LHβ: 0.13 ± 0.07%, TSHβ: 0.07 ± 0.00%, GH: 0.73 ± 0.23%, PRL: 0.09 ± 0.04%). The co-localization of α-MSH and SOX2 was also observed. As shown in Fig. 6D, although the hormone-positive rate among SOX2-positive cells was high for ACTH (4.55 ± 0.71%) and GH (3.20 ± 1.10%), other lineage cells showed low-value coexistence ratios (LHβ: 0.59 ± 0.32%, TSHβ: 0.39 ± 0.08%, PRL: 0.39 ± 0.19%). These results indicate that SOX2-positive cells exist as non-hormone-producing cells in the dairy cattle pituitary gland and can differentiate into all types of hormone-producing cells, in line with the results reported in the context of rodents.

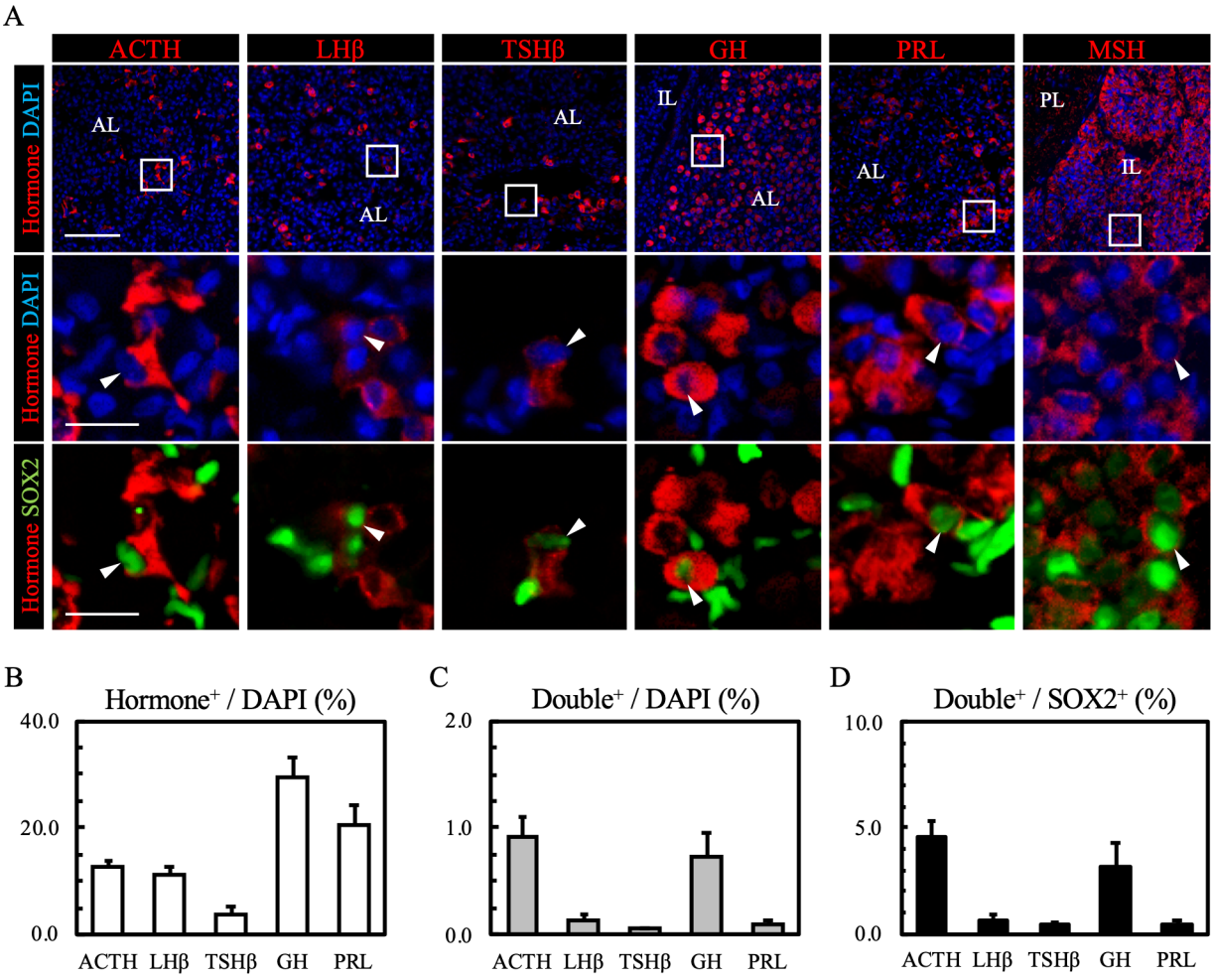

Fig. 6. Proportion of cells positive for sex-determining region Y-box 2 (SOX2) and hormones in the dairy cattle pituitary gland. (A) Double-immunostaining for SOX2 (green) and each pituitary hormone [adrenocorticotropic hormone (ACTH); luteinizing hormone β (LHβ); thyroid-stimulating hormone β (TSHβ); growth hormone (GH); prolactin (PRL); alpha-melanocyte-stimulating hormone (α-MSH)] (red) on the parenchyma of the anterior lobe in the dairy cattle pituitary gland was performed, together with DAPI (nuclei staining, blue). Merged images are shown in upper panels, and the boxed areas are enlarged in middle and lower panels. Arrowheads show SOX2/hormone double-positive cells. Scale bars=100 µm (upper panels), 20 µm (middle and lower panels). AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. (B–D) Numbers of cells positive for each protein in an area of 0.24 mm^2/section were counted, and the hormone-positive rate among total cells (Hormone$^+$ / DAPI), SOX2/hormone double-positive rate among total cells (Double$^+$ / DAPI), and SOX2/hormone double-positive rate among SOX2-positive cells (Double$^+$ / SOX2$^+$) were calculated. Data are shown as the means ± SD of four sections per hormone.

DISCUSSION

The fact that SOX2-positive cell subpopulations in rodents, located in both the MCL and parenchymal niches, have the proliferative activity and differentiate into hormone-producing cells, indicate that these cells exist as stem/progenitor cells in the pituitary gland. In the present study, we performed the immunofluorescence analysis for SOX2-positive cells in the pituitary gland of dairy cattle.

In the pituitary glands of adult rodents, the MCL (facing Rathke's cleft) and SOX2-positive dense cell clusters scattered throughout the parenchyma of the anterior lobe are proposed to act as primary and secondary stem/progenitor cell niches, respectively [4, 8, 14, 22]. The present study showed that SOX2-positive cells are widely distributed in the dairy cattle pituitary gland, especially in the MCL facing the lumen of the pouch and parenchymal cell clusters at high density. Furthermore, the stem/progenitor cell niche markers E-cadherin and CK8+18 are enriched in the MCL and parenchymal cell clusters. Our data are consistent with the findings in mice and rats [4, 8, 14], suggesting that SOX2-positive pituitary stem/progenitor cells are present across species, at least mammals, in the MCL and parenchymal niches. Additionally, we also found that some SOX2-positive cells co-localized with Ki67 and hormones. These results suggest that SOX2-positive cells in the dairy cattle pituitary gland can proliferate and differentiate into all types of hormone-producing cells. Furthermore, the proportion of hormone-producing cells in dairy cattle was relatively similar to that in rats after sexual maturity [10]. From this result, it is considered that the dairy cattle used in the present study have mature pituitary functions; 1-year-old Holstein heifers are already fertile [6].

Gene expression studies using rodent pituitary glands have demonstrated that pituitary stem/progenitor cells in the anterior lobe are composed of SOX2-positive cell subpopulations [1, 3, 7–9, 14, 18, 21]. The present study revealed that most of the SOX2-positive cells, located in the MCL of the dairy cattle pituitary gland, expressed PRRX1+2, similar to the observation in rats and mice [9, 14]. However, we identified a subpopulation of SOX2-positive pituitary stem/progenitor cells that do not express S100β in dairy cattle. Of note, we have recently demonstrated that SOX2/PRRX1 double-positive cells isolated from the MCL-niche of the mouse pituitary gland show high proliferation activity and differentiation capacity into hormone-producing cells and do not express *S100b* [14]. Furthermore, it has been reported that the majority of SOX2-positive cells in adult mouse and common marmoset pituitary glands are S100β-positive, and that the SOX2-positive/S100β-negative cell subpopulation is a minority [1, 19]. The finding that all SOX2-positive cells are S100β-negative may indicate a unique feature of bovine pituitary stem/progenitor cells. The results of the present study clearly suggest that SOX2-positive pituitary stem/progenitor cells might be conserved across rodents, common marmosets, and bovines, but main cell populations localized to the MCL-niche could be classified into several types depending on the animal species.

In summary, we demonstrated that SOX2-positive pituitary stem/progenitor cells in dairy cattle are localized especially in the stem/progenitor cell niche, the MCL, in line with the observation in mice [14]. Recently, the construction of artificial pituitary glands for use in regenerative medicine has become a reality via three-dimensional culture methods using embryonic stem cells, inducible pluripotent stem cells, and experimentally isolated pituitary stem/progenitor cells from humans and mice [5, 11, 13, 16]. However, this approach was not possible in the context of the bovine, since, thus far, their pituitary pluripotent cells were unknown. If the isolation of SOX2/PRRX1+2 double-positive pituitary stem/progenitor cells in dairy cattle can be achieved using our established method in mice [14], it will be an essential technique for investigating the supply of hormone-producing cells and tissue regeneration in bovine.

CONFLICT OF INTEREST. The authors declare no competing interests.

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