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—Original Article—

Colocalization of GPR120 and anterior pituitary hormone-producing cells in female Japanese Black cattle

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Abstract. Negative energy balance in domestic animals suppresses their reproductive function. These animals commonly use long-chain fatty acids (LCFAs) from adipocytes as an energy source under states of malnutrition. The G-protein coupled receptor, GPR120, is a specific receptor for LCFAs, but its role in reproductive function remains unknown in domestic animals. The purpose of this study was to examine whether GPR120 is involved in the reproductive system of cattle. *GPR120* mRNA expression was evaluated in brain, pituitary, and ovarian tissue samples by RT-PCR. *GPR120* gene expression was detected with high intensity only in the anterior pituitary sample, and GPR120-immunoreactive cells were found in the anterior pituitary gland. Double immunohistochemistry of GPR120 in the anterior pituitary hormone-producing cells, such as gonadotropes, thyrotropes, lactotropes, somatotropes, and corticotropes, was performed to clarify the distribution of GPR120 in the anterior pituitary gland of ovariectomized heifers. Luteinizing hormone β subunit (LHβ)- and follicle-stimulating hormone β subunit (FSHβ)-immunoreactive cells demonstrated GPR120 immunoreactivity at 80.7% and 85.9%, respectively. Thyrotropes, lactotropes, somatotropes, and corticotropes coexpressed GPR120 at 21.1%, 5.4%, 13.6%, and 14.5%, respectively. In conclusion, the present study suggests that GPR120 in the anterior pituitary gland might mediate LCFA signaling to regulate gonadotrope functions, such as hormone secretion or production, in cattle.
Key words: Gonadotrope, GPR120, Long-chain fatty acids (LCFAs), LCFA signaling, Pituitary

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Negative energy balance suppresses reproductive functions such as gonadotropin secretion, follicular development, fertilization, and early embryonic development in mammals as a survival strategy, because reproductive processes require extra energy. However, signaling molecules that translate peripheral undernutrition status for regulating reproductive function are not yet well known. In a state of undernutrition, blood glucose levels are low and circulating free fatty acid (FFA) levels are increased, and animals commonly start to manage fat mobilization. Indeed, animals that have been fasting, such as cows and sheep, showed a rise in plasma FFAs concentrations[1, 2]. It is thus conceivable that FFAs play a role in signaling a peripheral undernutrition status to the central nervous system governing reproductive function to save energy for reproduction. Therefore, it is useful to understand how FFAs control reproductive functions to resolve reproductive disorders in ruminants.

Gonadal functions of ruminants are controlled by the hypothalamic-pituitary-gonadal (H-P-G) axis similarly to that in other mammals. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretions from gonadotropes of the anterior pituitary gland are stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. Under the negative energy state, the suppression of gonadotropin secretion has been observed in various species. For example, nutritionally restricted goats [3] and sheep [4] became hypogonadotropic, exhibiting a low frequency of pulsatile LH secretion. Further, 24 and 48 h of fasting inhibited pulsatile LH secretion in mice [5] and rats [6], respectively. In these previous studies, gonadal function was thought to be regulated at the hypothalamic level of the H-P-G axis. However, recent studies suggest that gonadal function is controlled not only by the hypothalamus but also at the pituitary level. For instance, cortisol directly suppressed pulsatile LH secretion from the pituitary gland in sheep [7]. Adiponectin, leptin, and other hormones are known to regulate LH secretion directly via gonadotropes

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[8–10]. Further, an administration of long-chain fatty acids (LCFAs) directly reduced luteinizing hormone β subunit (*Lhb*) and follicle-stimulating hormone β subunit (*Fshb*) gene expression in LβT2 mice pituitary cell line [11]. This indicates that the synthesis and secretion of gonadotropins, LH and FSH can be directly regulated at the pituitary level by peripheral signals in mammals, including ruminants.

G-protein coupled receptor, GPR120, functions as a specific receptor for LCFAs [12]. GPR120 is a lipid sensor in the control of energy balance in human and mice, given that *Gpr120* gene-deficient mice develop obesity and obese subjects harbor a deleterious non-synonymous mutation in the *Gpr120* exon that inhibits GPR120 signaling [13]. A previous study showed that *Gpr120* mRNA is strongly expressed in the pituitary gland, lung, small intestine, colon, and adipose tissues of male mice [14]. Interestingly, Moriyama *et al*. [15] detected GPR120-immunoreactive cells in the anterior pituitary gland and showed that 24 h fasting increased *Gpr120* gene expression in the pituitary gland of mice. This raises a possibility that pituitary GPR120 is involved in controlling reproductive functions by sensing the levels of circulating LCFAs. However, previous studies on pituitary GPR120 is limited to mice and cell lines, and the physiological functions of GPR120 in the pituitary gland have not yet been fully elucidated. Importantly, understanding a central mechanism that reflects metabolic status to reproductive functions in cattle would be helpful for the reproduction of domestic animals that are undernutrition.

The central mechanism underlying nutritional restriction on reproductive function in cattle has yet to be revealed, while the reproductive issues caused by nutritional condition is a topic of increasing importance in livestock. The present study focused on the localization of GPR120 in the anterior pituitary gland of cattle, aiming to reveal the roles of GPR120 in anterior pituitary hormones, i.e. LH, FSH, thyroid-stimulating hormone (TSH), prolactin (PRL), growth hormone (GH), and adrenocorticotropic hormone (ACTH). First, we investigated *GPR120* gene expression within the H-P-G axis by RT-PCR on brain, pituitary, and ovarian tissues of cattle. We then performed immunohistochemistry of GPR120 in the pituitary gland of heifers. In accordance with the gene and protein expression, we then examined the distribution of GPR120 by dual immunohistochemistry for each anterior pituitary hormone in the anterior pituitary gland of heifers.

Materials and Methods

Animals and experimental procedure

Japanese Black heifers (n = 3) used for histological analysis and adult female Japanese Black cattle (n = 1) used for gene expression analysis of *GPR120* in the pituitary were kept at the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NARO-ILGS). These animals were housed under natural conditions and provided grass silage and water ad libitum. Adult female Japanese Black cattle (n = 2) in a slaughterhouse were used for gene expression analysis of *GPR120* in the brain and ovary. Japanese Black heifers used for histological analysis were ovariectomized at least 1 month before tissue collection to detect strong staining signals of GPR120 on the tissues, as ovariectomies increased pituitary *Gpr120* gene expression in mice [16]. All experimental procedures involving animals were approved by the Committee of the Care and Use of Experimental Animals of NARO-ILGS, Japan.

Tissue sampling for gene expression analysis

The pituitary sample for gene expression analysis was collected from deeply anesthetized female Japanese Black cattle that was killed by sodium pentobarbital overdose and potassium chloride solution in NARO-ILGS. The sample was separated to anterior, intermediate, and posterior pituitary glands, respectively. Brain samples for gene expression analysis were collected from female Japanese Black cattle at a slaughterhouse. The hypothalamus, cerebral cortex, and hindbrain were collected, and hypothalamic tissues were divided into anterior and posterior parts, including the preoptic area (POA) and the arcuate nucleus (ARC), respectively, because GnRH and kisspeptin neurons that are responsible for reproductive function are located in the POA and the ARC in cattle [17, 18]. Brainstem sample included area postrema containing ependymocytes of the wall of forth cerebral ventricle that play a role for sensing nutrition condition [19, 20]. The ovary was collected from slaughtered Japanese Black cattle and separated into stroma, granulosa cells, and corpus luteum, respectively. Samples that weighed 100 mg or less were immediately incubated in 900 µl of QIAsol Lysis Reagent (QIAGEN, Hilden, Germany) according to the manufacture's instruction. The samples were homogenized on ice, and then stored at –80°C until total RNA extraction was performed. Total RNA was extracted using RNeasy Plus Universal Mini Kit (QIAGEN) and cDNA from each sample was synthesized with ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. Gene expression of *GAPDH* and *GPR120* was analyzed by PCR using the following primer sets: *GAPDH* (NM_001034034) forward, 5'-AGT TCA ACG GCA CAG TCA AG-3'; reverse 5'-CAT ACT CAG CAC CAG CAT CA-3', *GPR120* (NM_001328657) forward, 5'-AGG AAC GAA TGG AGG TT-3'; reverse, 5'-GTG CTG AGG GTC ATG GAA AT-3'. PCR was carried out with Ex Taq DNA polymerase (TaKaRa, Shiga, Japan) using the following program: 94°C for 5 min, 30 cycles of 98°C for 30 sec, 60 or 62°C for 30 sec and 72°C for 30 sec, and 72°C for 7 min. DNA was visualized by ethidium bromide after electrophoresis.

Immunohistochemistry

The pituitary glands of Japanese Black heifers for histological analysis were collected after perfusion with 10 l of phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA). The samples were post-fixed with 4% PFA overnight at 4°C and were placed in 20% sucrose until tissues had sunk. The tissues were cut into 6 µm of sagittal sections by cryostat (CM1850; Leica, Wetzlar, Germany) using Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan), and then sections were mounted on silane-coated slide glasses. Slides were boiled for 30 min in 0.01 M sodium citrate (pH 6.0) for antigen retrieval in order to use immunohistochemistry. Sections were washed using PBS with 0.1% Tween 20 (PBST) followed by incubation with 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity. Next, sections were incubated with blocking solution of PBST containing 10% ImmunoBlock (DS Pharma Biomedical, Osaka, Japan) for 1 h followed by an overnight incubation with primary antiserum against GPR120 (1:10000) [21] at room temperature. For a single label immunohistochemistry

of GPR120, sections were incubated for 2 h in biotinylated goat anti-rabbit IgG antibody (1:200, BA-1000, Vector Laboratories, Burlingame, CA, USA). After washing in PBST, the sections were incubated for 1 h in ABC reagent (PK-4000, Vector Laboratories). Thereafter, immunoreactivities were visualized with 0.05% diaminobenzidine and 0.006% H_2O_2 in 0.1 M Tris-HCl buffer. For a double label immunohistochemistry, immunoreactivities of GPR120 were visualized by the TSA Biotin System (PerkinElmer, Brachburg, NJ, USA) using Alexa 555 conjugated streptavidin (1:800; Life Technologies, Palo Alto, CA, USA). These sections were incubated with antiserum against LHβ, FSHβ, TSHβ, PLR, GH and ACTH, respectively as described in Table 1, overnight at room temperature, and then, Alexa 488 conjugated IgG for 2 h. Finally, the sections were embedded with Prolong Gold containing 4',6-diaminodino-2-phenylinodole (DAPI; Life Technologies) for nuclear staining. Removal of primary antibodies from the immunohistochemical protocol results in complete absence of signals. The specificity of anti-human GPR120 antibody was confirmed by preincubation of the antiserum with 20 μg synthetic cattle GPR120 peptide (PH Japan, Hiroshima, Japan) overnight at 4°C. The specificity of anti-LHβ, FSHβ, TSHβ, PRL, GH, and ACTH antibodies were confirmed by preincubation of antiserum with 200 μg bovine pituitary extract (Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4°C. Since the available primary antibodies recognizing GPR120 and hormones were raised in rabbits, previous protocols described by Hunyady *et al*. [22] and Amstalden *et al*. [23] were used to eliminate the possibility of cross reactivity and false colocalization of the target. Briefly, the GPR120 was visualized by a very low concentration of antibody followed by TSA amplification system. Next, the primary antibodies against hormones were visualized by indirect detection of fluorescence conjugated secondary antibody. This procedure was reported in previous studies [24, 25]. Micrographs were obtained using a fluorescent microscope (FSX100, Olympus, Tokyo, Japan).

Quantification of GPR120-immunopositive cells in hormone-positive cells

The colocalization ratio of GPR120 in each hormone-immunopositive cell was calculated by dividing the number of double-labeled neurons by the total number of hormone-expressing cells. Data were averaged using the ratio in 3 locations (anterior, medial and posterior parts) from each of 3 nonconsecutive unilateral sections obtained from 3 animals.

Results

GPR120 gene and protein expressions in the pituitary gland of cattle

Reverse-transcription PCR analysis demonstrated *GPR120* gene expression in the anterior pituitary gland in cattle (Fig. 1). Low band intensity was observed in the posterior pituitary gland and granulosa cell. However, *GPR120* gene expression was not detected in brain samples, the intermediate pituitary gland, ovarian stromal cells, and corpus luteum. Immunohistochemistry with anti-human GPR120 antibody revealed that GPR120-immunopositive cells are located in the anterior pituitary gland and not in the intermediate or the posterior pituitary gland (Fig. 2C, D, E, F).

Characterization of GPR120-immunopositive cells in the anterior pituitary gland of heifers

Colocalization of GPR120-immunopositive cells and anterior-pituitary hormone examined by double-label immunohistochemistry. GPR120-immunoreactivities were observed in almost all LHβ- and FSHβ-producing cells (80.7% and 85.9%, respectively; Table 2, Fig. 3). On the other hand, TSHβ-, PLR-, GH- and ACTH-producing cells coexpressing GPR120 protein were limited (21.1%, 5.4%, 13.6% and 14.5%, respectively; Table 2, Fig. 3). No immunoreactivity was found with preabsorbed antibodies (Fig. 4).

Discussion

The present study demonstrates *GPR120* gene and protein expressions in the anterior pituitary gland of cattle, and that the GPR120 protein was especially localized to LHβ and FSHβ producing cells. To our knowledge, this is the first demonstration in livestock animals that GPR120 is mainly localized in the gonadotropes of the cattle pituitary gland. Our findings would be useful in ruminant livestock because ruminants are often malnourished during the postpartum period, and earlier restoration of the estrus cycle is needed to improve the reproductive performance. Gonadotrope-specific high expression of GPR120 would provide a possible mechanism for sensing LCFAs at the pituitary level. Previously, GnRH-induced LH secretion was inhibited by LCFAs treatment in primary pituitary cell culture from mice [26] and rats [27] and a low dose of unsaturated fatty acids reduced the transcriptional activity of the *Fshb* gene [11]. GPR120 has been recognized as a lipid sensor in mice and humans through a loss of function study [13]. These findings and gonadotrope-specific

Table 1. Informaiton of primary antibody for immunohistochemistry

Antibody	Host	Type	Dilution	Source
anti-human GPR120	Rabbit	Polyclonal	1:10000	Miyauchi *et al*., 2009 [21]
anti-ovine LHβ	Rabbit	Polyclonal	1:5000	NIDDK, CA, USA
anti-ovine FSHβ	Rabbit	Polyclonal	1:1000	NIDDK, CA, USA
anti-rat TSHβ	Rabbit	Polyclonal	1:1000	NIDDK, CA, USA
anti-mouse PRL	Chicken	Polyclonal	1:5000	Donated by Dr. Toshio Harigaya, Meiji University, Japan
anti-rat GH	Rabbit	Polyclonal	1:1000	Donated by Dr. Masayuki Iigo, Utsunomiya University, Japan
anti-rat ACTH	Rabbit	Polyclonal	1:1000	NIDDK, CA, USA

NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases.

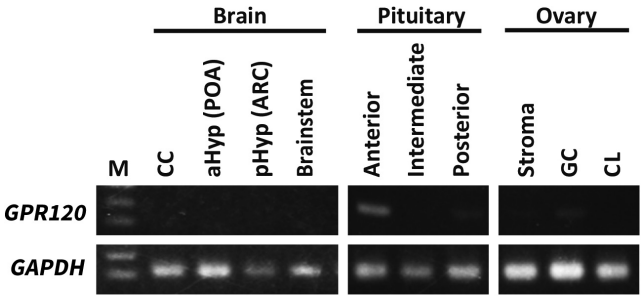

Fig. 1. Expression of *GPR120* mRNA in brain, pituitary, and ovarian tissues of cattle. Electrophoresis imaging shows amplification products of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, 118 bp) and *GPR120* (196 bp) genes. *GAPDH* was used as the internal control. Upper and lower molecular markers show 200 and 100 bp, respectively. M, DNA marker; CC, cerebral cortex; aHypo, anterior part of hypothalamus; pHypo, posterior part of hypothalamus; GC, granulosa cell; CL, corpus luteum.

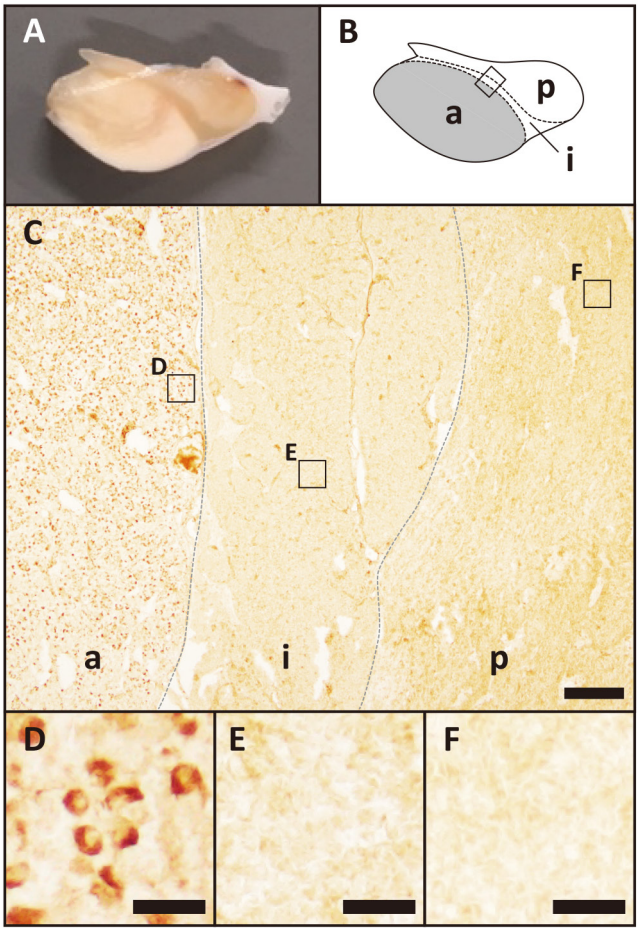

Fig. 2. GPR120 protein expression in the anterior pituitary gland of heifer. Sagittal photo image (A) and illustration (B) of pituitary gland of heifer. GPR120 immunoreactivity (brown) in pituitary gland (C) that is indicated in square in B. Scale bar = 100 µm. Magnification images of the anterior (D), the intermediate (E), and the posterior (F) pituitary gland are indicated in square in C. Scale bar = 25 µm. a, anterior pituitary gland; i, intermediate pituitary gland; p, posterior pituitary gland.

expression patterns of GPR120 in mice [15] and cattle (present study) suggest that LCFAs-GPR120 signaling might control reproductive function. Further, twenty-four hours of fasting induced an increase in the levels of circulating FFAs in various species [1, 2, 28]. Fasting also induced an elevation of *Gpr120* mRNA expression in the pituitary gland of mice [15]. Suppression of LH secretion has been reported in various species with negative energy status [3–6], namely high levels of circulating FFAs. Taken together, an increase in LCFAs levels caused by fasting might activate GPR120 signaling in gonadotropes, which may be reflected in their functions, such as *Lhb* and *Fshb* mRNA expression or gonadotropin secretion *in vivo*. Thus, GPR120 in gonadotropes may act as a sensor of circulating LCFA levels in order to control gonadotropin synthesis and secretion, especially in malnourished cattle. Further research is required to uncover the physiological function of GPR120 in gonadotropes.

Importantly, though GPR120 is a well-known Gq-coupled GPCR, recent evidence has demonstrated that it may act via activation of the Gi pathway to inhibit ghrelin [29] and somatostatin [30] secretions. It is notable that palmitate suppressed intracellular cAMP levels in MGN3-1, a ghrelin-producing cell line, which is known to express GPR120 [31]. Moreover, treatment with GW-9508, a GPR120 agonist, inhibited ghrelin secretion *in vitro* and *in vivo* [32]. Further studies are needed to clarify the mechanism of GPR120 signaling in gonadotropes, particularly the associated G protein pathway.

The high percentage of colocalization of GPR120 and gonadotropic cells in heifers was consistent with that in a previous study conducted in mice, in which almost all LHβ- and FSHβ-producing cells coexpressed GPR120 protein in the anterior pituitary gland [15]. These results imply that the GPR120 expression pattern in gonadotropes is highly conserved at least between mice and cattle. This study also showed that other anterior pituitary hormone-producing cells, such as TSHβ-, PRL-, GH- and ACTH-producing cells, partly coexpress GPR120 in heifers. Interestingly, no GPR120-immunoreactive cells were found in anterior pituitary hormone-producing cells, except for gonadotropes in mice [15]. These data suggest that localization

Table 2. Immunohistochemical colocalization of GPR120 and pituitary hormones in the anterior pituitary gland of ovriectomized heifers

	LHβ	FSHβ	TSHβ	PRL	GH	ACTH
% hormone-immunoreactive cells co-expressing GPR120	80.7 ± 2.2	85.9 ± 4.7	21.1 ± 14.1	5.4 ± 0.4	13.6 ± 0.7	14.8 ± 4.8

LHβ, luteinizing hormone β subunit; FSHβ, follicle-stimulating hormone β subunit; TSHβ, thyroid-stimulating hormone β subunit; PRL, prolactin; GH, growth hormone; ACTH, adrenocorticotropic hormone.

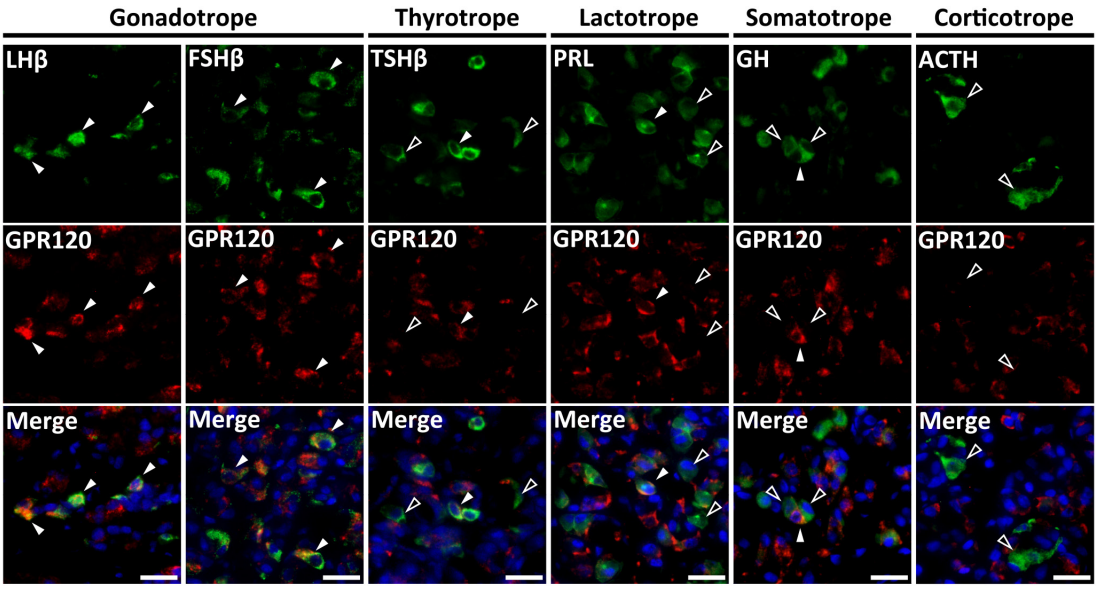

Fig. 3. Dual immunohistochemistry of GPR120 (red) and luteinizing hormone β subunit (LHβ) (green), follicle-stimulating hormone β subunit (FSHβ) (green), thyroid-stimulating hormone β subunit (TSHβ) (green), prolactin (PRL) (green), growth hormone (GH) (green), and adrenocorticotropic hormone (ACTH) (green) with 4',6-diaminodino-2-phenylinodole (DAPI) (blue) in anterior pituitary gland of ovariectomized heifers. White and black arrowheads indicate representative dual-labeled cells and single-labeled cells, respectively. Scale bar = 30 μm.

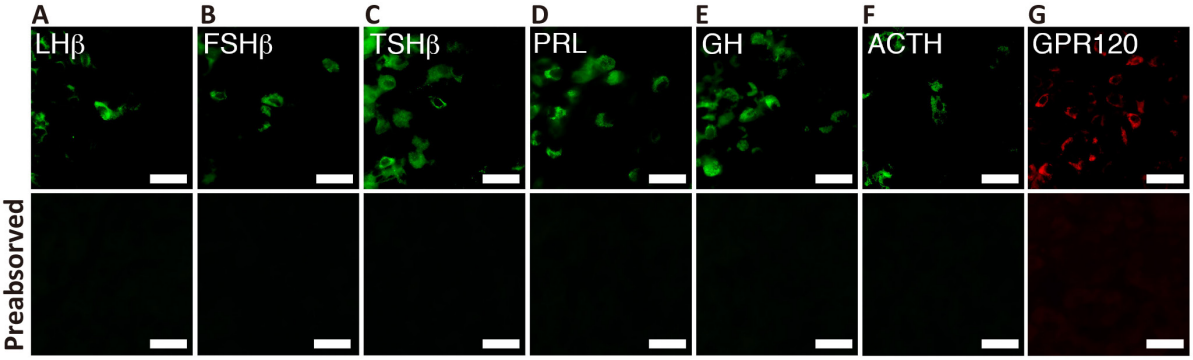

Fig. 4. Specificity of primary antibodies in the anterior pituitary gland of heifer. Luteinizing hormone β subunit (LHβ) (A), follicle-stimulating hormone β subunit (FSHβ) (B), thyroid-stimulating hormone β subunit (TSHβ) (C), prolactin (PRL) (D), growth hormone (GH) (E), adrenocorticotropic hormone (ACTH) (F), GPR120 (G) immunoreactivity was eliminated by preincubation of primary antibody with bovine pituitary extract or synthetic antigen peptide in heifer pituitary tissue. Scale bar = 30 μm.

of GPR120 in hormone-producing cells other than gonadotropes might have species-specific differences. This raises the possibility that hormone-producing cells coexpressing GPR120 are regulated by LCFAs via GPR120. Since the expression rates of GPR120 in thyrotropes, lactotropes, somatotropes, and corticotropes were quite low compared to those in gonadotropes, further studies are needed to confirm the physiological significance of LCFAs-GPR120 signaling for regulating those hormone-producing cells in the anterior pituitary gland.

This study also showed that *GPR120* gene is expressed in the posterior pituitary gland and granulosa cells of cattle. To our knowledge, very few to no studies have previously focused on GPR120 in the posterior pituitary gland. Additionally, the relationship between GPR120 signaling and hormones, such as oxytocin and vasopressin that project from the hypothalamus, remains unknown. Further studies are required to know the function of GPR120 in the posterior pituitary gland. In ovaries, a previous study showed that GRP120 protein express bovine granulosa cells; and docosahexaenoic acid (DHA), a LCFA, was shown to modulate the proliferation and steroidogenesis of these granulosa cells [33]. The authors found no *GPR120* gene expression in the hypothalamus, cerebral cortex, and brainstem. A previous study showed that undernourished state,

namely hypoglycemia, inhibits GnRH/LH secretion through the glucose-sensing system located in the lower brain stem in rats [34]. Consequently, the lower brainstem is one of the energy-sensing regions that regulate gonadal functions in mammals. Nevertheless, in the present study, GPR120 expression was not observed in the lower brainstem. It is likely that the metabolic sensing system that regulates gonadal functions is different between the lower brainstem and the pituitary gland.

In summary, the present study clearly indicates that *GPR120* gene and protein expression occur in the anterior pituitary gland, particularly in gonadotropes of heifers. Further studies focusing on a physiological role of GPR120 in the pituitary gland are needed to clarify whether negative energy status is sensed at the pituitary level to modulate reproductive function.

Conflict of Interests: The authors declare no conflicts of interest associated with this manuscript.

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