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—Original Article—

Transcription of follicle-stimulating hormone subunit genes is modulated by porcine LIM homeobox transcription factors, LHX2 and LHX3

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Abstract. The LIM-homeobox transcription factors LHX2 and LHX3s (LHX3a and LHX3b) are thought to be involved in regulating the pituitary glycoprotein hormone subunit genes *Cga* and *Fshβ*. These two factors show considerable differences in their amino acid sequences for DNA binding and protein-protein interactions and in their vital function in pituitary development. Hence, we compared the DNA binding properties and transcriptional activities of *Cga* and *Fshβ* between LHX2 and LHX3s. A gel mobility shift assay for approximately 1.1 kb upstream of *Cga* and 2.0 kb upstream of *Fshβ* varied in binding profiles between LHX2 and LHX3s. DNase I footprinting revealed DNA binding sites in 8 regions of the *Cga* promoter for LHX2 and LHX3s with small differences in the binding range and strength. In the *Fshβ* promoter, 14 binding sites were identified for LHX2 and LHX3, respectively. There were alternative binding sites to either gene in addition to similar differences observed in the *Cga* promoter. The transcriptional activities of LHX2 and LHX3s according to a reporter assay showed cell-type dependent activity with repression in the pituitary gonadotrope lineage LβT2 cells and stimulation in Chinese hamster ovary lineage CHO cells. Reactivity of LHX2 and LHX3s was observed in all regions, and differences were observed in the 5'-upstream region of *Fshβ*. However, immunohistochemistry showed that LHX2 resides in a small number of gonadotropes in contrast to LHX3. Thus, LHX3 mainly controls *Cga* and *Fshβ* expression.
Key words: Gene regulation, Glycoprotein hormone, LHX2, LHX3, LIM-homeodomain, Pituitary

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The pituitary gonadotropin hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are dimeric proteins composed of a common glycoprotein hormone α subunit (Cgα) and a unique β subunit (FSHβ and LHβ) that confers biological specificity for their respective hormone functions. The regulatory mechanisms of the three subunit genes constituting two types of gonadotropins are of special interest because three subunit genes are modulated in the same cell with distinct roles in gametogenesis in both sexes. To resolve gene regulation on the molecular level, several approaches have been used [1] and have identified many regulatory factors and elements governing the basal and cell-specific expression of the subunit gene [2]. We have more recently reported a novel regulation with long-fatty acid for *Fshβ* [3].

Among the various types of transcription factors known to participate in the control of gonadotropin genes, LIM homeobox 3 (LHX3, also known as P-Lim/LIM-3) plays a crucial role in pituitary development [4, 5]. *Lhx3*-knockout mice showed that this gene is essential for early pituitary structural development and later for the differentiation of cell types in the anterior and intermediate lobes. LHX3 also participates in the activation of hormone gene expression, either alone or in synergy with other regulatory factors [6–9]. Subsequently, LHX3 was identified as a regulator of porcine *Fshβ* [10]. In contrast, LHX2 (also known as LH2), which belongs to the same subfamily of LHX3, plays an important role in eye, forebrain, and definitive erythrocyte development [11]. *Lhx2*-knockout mice showed a defect in the posterior lobes that induced the disorganization of the anterior/intermediate lobes with differentiation of their hormone-producing cells [12]. LHX2 was first identified as a regulatory factor for *Cga* in the pituitary tumor-derived cell line αT3-1 [13]. More recently, we cloned *Lhx2* cDNA from the porcine anterior pituitary cDNA library using the Yeast One-Hybrid Cloning System and the upstream region of *Fshβ* as a bait sequence. The results demonstrated that LHX2 modulates porcine *Fshβ* by binding to plural sites of the promoter [14].

LIM homeobox transcription factors are characterized as having two LIM domains and a homeobox domain. LIM domains are

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known to have highly divergent sequences, providing many different binding properties and forming various combinations [15]. The homeobox domain is composed of 60 amino acids that form three α-helixes characterized by their DNA binding properties as well as dimerization [16–19]. However, differences in the residues important for DNA binding or dimerization alter binding specificity or the loss of dimerization [20–22]. LHX2 and LHX3 show a similarity of less than about 50%, indicating that their DNA binding and/or transcriptional regulation activities also differ.

Considering the dissimilarity in the domain sequences, the present study aimed to compare the DNA binding properties and transcriptional activities of LHX2 and LHX3. In addition, because different isoforms of human LHX3 have been reported [9] but those in porcine species remain unknown, we cloned their full-length cDNAs. As expected, LHX2 and LHX3 showed some differences in their DNA binding abilities in their strengths and specificities as well as in their regulatory potencies for several promoter regions of both genes. Immunohistochemical analysis, however, demonstrated a rare population of LHX2 in contrast to LHX3 in FSHβ-positive cells. The present study showed that LHX2 and LHX3 differentially regulate *Fshβ* and *Cga*.

Materials and Methods

Cloning of porcine LIM-homeodomain transcription factor Lhx3

The amino terminus of the porcine *Lhx3a* and *Lhx3b* was first determined using a primer set by PCR for the porcine pituitary cDNA library [23]. Next, the forward primers for the amino terminus of *Lhx3a* (5′- GGGGAATTCGCCATGCTGCTGGAAACGGAGCTG-GCGGG-3′) and *Lhx3b* (5′-GGGGAATTCGCCATGGAAGCG-CGCGGGGAGCTG-3′) were synthesized and used to amplify their full-length cDNAs together with a common reverse primer (5′-TCCTCGAGCTGGG GCCTCAGTCAGAACTG-3′), followed by confirmation of the nucleotide sequence of the amplified DNAs.

Construction of vectors for production of recombinant protein, expression in mammalian cells, and promoter assay

The full-length expression vector of porcine *Lhx2*, *Lhx3a*, *Lhx3b*, and *Lhx3-del* (consisting of a 28–406 amino acid region with a primer 5′-GAGAATTCGCGATGGA TCCCACTGTGTGCC-3′ based on a previous paper [24]) was constructed in the mammalian expression vector, pcDNA3.1/Zeo+ (Invitrogen, Carlsbad, CA, USA). For the DNA-binding assay, recombinant proteins were produced by cloning of the truncate cDNAs, *ΔLIM-Lhx2* (ΔLIM-LHX2 consisting of amino acid numbers 170–406) and *ΔLIM-Lhx3* (ΔLIM-LHX3 consisting of 148–401), into the pET32a vector, followed by expression in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL (Stratagene, La Jolla, CA, USA), since the LIM domains aggregate by interacting as described previously [25]. The recombinant proteins were expressed and prepared using an Overnight Express Autoinduction System 1 (Novagen, Madison, WI, USA), followed by purification using His-Tag Mag beads (Toyobo, Osaka, Japan).

The following vectors were constructed for the promoter assay in pSEAP-Basic (Clontech Laboratories, Mountain View, CA, USA; αGSU (−1059/+12), αGSU (−798/+12), αGSU (−551/+12), αGSU (−239/+12), and αGSU (−100/+12) for *Cga* [13] and FSHβ (–1965/+10), FSHβ (–985/+10), FSHβ (–238/+10), and FSHβ (–103/+10) for *Fshβ* [14], respectively.

Electrophoretic mobility shift assay (EMSA) and DNase I footprinting assay

The production of FAM-labeled DNA fragments was conducted as described previously [26]. EMSA and DNase I footprinting were also carried out as previously described [23, 27].

Cell culture, transfection, and promoter assay

A transient transfection assay was carried out using LβT2 cells and Chinese hamster ovary cells (CHO) as previously described [28]. LβT2 cells, which were kindly provided by Dr PL Mellon (University of California, San Diego, CA, USA), are a mouse pituitary gonadotrope lineage cell line [29] that endogenously expresses the gonadotropin genes *Cga*, *Lhβ*, and *Fshβ* [30]. CHO cells are a non-endocrine cell line. After incubation for more than 48 h, an aliquot (5 μl) of cultured medium was assayed for secreted alkaline phosphatase activity using the Phospha-Light Reporter Gene Assay System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany). All values were expressed as the mean ± SD of quadruplicate transfections in two independent experiments. The reproducibility and reliability of the reporter assay without an internal control were conducted as described in our previous paper [31]. Statistical significance was calculated by Dunetts' test with the F-test.

Quantitative real-time RT-PCR

Total RNAs, which were extracted from the porcine anterior pituitaries of German Landrace pigs with intact gonads of both sexes during the fetal (f40, f50, f65, f82, f95, and f110) and postnatal periods (p8, p60, p160 (prepuberty), and p230 (sexually matured)), were kindly supplied by Dr F Elsaesser and pooled for 1–6 individuals of the respective age and sex for cDNA synthesis as previously described [32].

Quantitative real-time PCR was performed using a specific primer set (Supplementary Table 1: online only) in duplicate with the same threshold line for *Lhx3a, Lhx3b*, and *cyclophilin A* (used as an internal control), and data were evaluated using the comparative C_T method ($\Delta\Delta C_T$ method) as previously described [14]. The DNA sequences of the PCR products were confirmed.

Immunohistochemistry

The pituitaries on postnatal day (P) 15 from S100β-GFP rats [33] were fixed with 4% paraformaldehyde in 20 mM HEPES, pH 7.5, overnight at 4°C, followed by immersion in 30% trehalose in 20 mM HEPES for tissue cryoprotection. Samples were embedded in Tissue-Tek O.C.T compound (Sakura Finetek Japan, Tokyo, Japan) and frozen immediately. Frozen sections (6-μm-thick) from the coronal plane were prepared. After washing with 20 mM HEPES-100 mM NaCl, pH 7.5 (HEPES buffer), these sections were reacted with primary antibodies at appropriate dilutions with 10% (v/v) fetal bovine serum and 0.4% (v/v) Triton X-100 in HEPES buffer overnight at room temperature. Primary antibodies used were rabbit IgG against mouse LHX2 (1:200 dilution, kindly provided by Dr

Es Monuki at University of California [34], rabbit IgG against mouse LHX3 (recognizes both LHX3a and LHX3b; 1:250 dilution, Abcam, Cambridge, UK), and chicken IgY against jellyfish GFP (Aves Labs, Tigard, OR, USA). Guinea pig antisera against the pituitary hormones rat FSHβ (1:40,000 dilution), rat PRL (1:10,000 dilution), and rat TSHβ (1:100,000 dilution) were kindly provided by the National Institute of Diabetes and Digestive and Kidney courtesy of Dr AF Parlow. Guinea pig antiserum against rat ACTH (1:10,000 dilution) and rat GH (1:6,000 dilution) were produced and kindly provided by Dr S Tanaka of Shizuoka University (Shizuoka, Japan). After the immunoreaction, the sections were washed with HEPES buffer and then incubated with secondary antibodies using Cy3- or Cy5-conjugated AffiniPure donkey anti-rabbit, guinea pig IgG and chicken IgY (1:500 dilution; Jackson ImmunoResearch, West Grove, PA, USA). The sections were washed with HEPES buffer and then enclosed in VECTASHIELD Mounting Medium with 4′, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was observed by fluorescence microscopy using a BZ-8000 microscope (KEYENCE, Osaka, Japan).

Results

Cloning of porcine Lhx3 isoforms

We determined the complete sequence of porcine LHX3 isoforms, as only partial sequences of the N-terminus of the two isoforms have been reported [24]. First, a common reverse primer was employed to determine the nucleotide sequences of the two isoforms, followed by amplification of the full-length isoforms (*Lhx3a* and *Lhx3b*) using specific forward primers containing the start codon ATG for translation. The nucleotide sequences revealed that the amino acid sequences were 401 and 403 residues for LHX3a and LHX3b, respectively (Supplementary Figs. 1 and 2: online only; sequences were deposited to DDBJ as Accession No. **AB797327** and **AB797328**). The amino acid Arg at position 26 of LHX3a differed from the previously identified residue Pro [24], while other N-terminal sequences of LHX3a and LHX3b were identical.

The similarity of the amino acid sequences between LHX2 and LHX3 was examined, as shown in Fig. 1. Two LIM domains, which are known to be important in protein-protein interactions, showed only 51 and 47% identities. Additionally, the homeobox domain, which is important for DNA-binding, showed a very low similarity of 43%, indicating that the two cognate proteins have different roles in gene regulation.

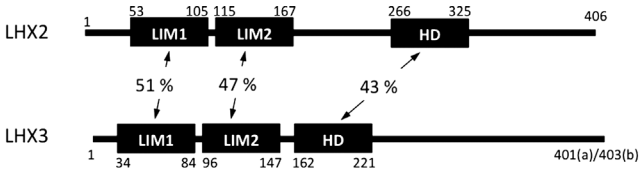

Fig. 1. Comparison of structures of porcine LHX2 and LHX3. LIM-homeodomain transcription factors are composed of two LIM domains (LIM1 and LIM2) and a DNA-binding homeodomain (HD), with numbered amino acid positions (LHX2 and LHX3a). LHX3a and LHX3b are composed of 401 (a) and 403 (b) amino acids, respectively. The homology (%) of the domains is indicated between the diagrams.

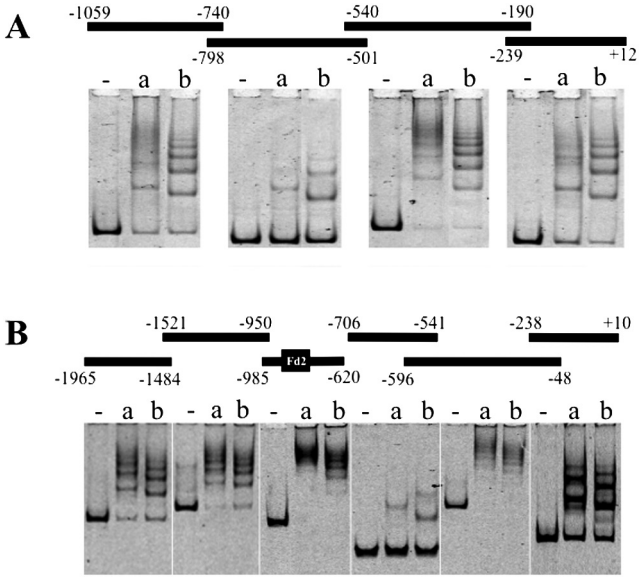

Fig. 2. Electrophoretic gel mobility shift assay of LHX2 and LHX3. Upstream fragments of *Cga* (A) and *Fshβ* (B) are indicated with a line, (thick bar in B indicates Fd2) and nucleotide number above the electrogram. The mixture without protein (-) or with recombinant LIM domain-deleted LHX2 (ΔLIM-LHX2; a) and LHX3 (ΔLIM-LHX3; b) proteins and FAM-labeled fragments were analyzed on a 4% polyacrylamide gel followed by visualization with a fluorescence viewer.

EMSA and DNase I footprinting

EMSA was carried out for the FAM-labeled upstream fragments of *Cga* and *Fshβ*. The results showed remarkable multiple-shift bands, except for weak bands for the –798/–501 bp fragment of *Cga* (Fig. 2A) and for the –706/–541 bp fragment of *Fshβ* (Fig. 2B). All shift band patterns differed between ΔLIM-LHX2 and ΔLIM-LHX3.

DNase I footprinting analyses were performed to determine the binding sequence, and the profiles with or without ΔLIM-LHX2 or ΔLIM-LHX3 were compared. Protection from DNase I digestion was observed in several regions of the *Cga* (Supplementary Fig. 3A: online only) and *Fshβ* (Supplementary Fig. 3B) promoters. We identified 8 binding sites in both ΔLIM-LHX2 and ΔLIM-LHX3 upstream of *Cga* (Table 1). The binding sites of ΔLIM-LHX2 were found at 14 positions, which agree with the results of our previous study [14], while ΔLIM-LHX3 showed 14 binding sites upstream of *Fshβ* (Table 2). DNase I footprinting analyses profiles were similar but showed differences in the binding strength and range in each region, which were comparable to the differences observed on the EMSA profiles (Fig. 2). The 6 binding sequences on *Cga* and 13 on *Fshβ* contained the nucleotide sequence TAAT/ATTA (Tables 1 and 2), which is known as a core binding motif for homeodomain transcription factors [16–19].

Table 1. Binding sites for LHX3 and LHX2 in the porcine *Cga* promoter

LHX3		LHX2
−1053/−1029	5'-GAAGaTGaTACTAATTCaTAT-3'	−1053/−1029
−941/−931	5'-AGAAATCAACT-3'	−941/−931
−922/−916	5'-ATAATAA-3'	−922/−910
−893/−890	5'-TTTG-3'	−893/−890
−493/−483	5'-TCCTTATTAAA-3'	−494/−483
−471/−439	5'-AAATATAATTtaCA-3'	−471/−439
−340/−326	5'-TATAATCA-3'	−340/−329
−132/−124	5'-ATGGTAATT-3'	−137/−119

Table 2. Binding sites for LHX3 and LHX2 in the porcine *Fshβ* promoter

LHX3		LHX2
−1914/−1899	5'-TCCaTTCaTTtGTGT-3'	−1915/−1899
−1882/−1865	5'-ATATAATTGTAATCATAT-3'	−1880/−1868
−1799/−1787	5'-TTGACAATTACT-3'	−1799/−1768
−1441/−1429	5'-CATGCCAATTATA-3'	−1441/−1422
−1166/−1145	5'-AATCAGATTCTTTGATTATTT-3'	−1156/−1145
−836/−826	5'-TAATTAATT-3'	−836/−827
−820/−810	5'-TTAATTAATTG-3'	−818/−810
−808/−798	5'-TCAATTAaTA-3'	−808/−798
−467/−454	5'-AAATATAATTtaCA-3'	−466/−454
−440/−433	5'-TATAATCA-3'	−440/−433
−380/−369	5'-GTAACTTATTAACC-3'	−380/−367
−301/−287	5'-TCCCCAAATTAAAT-3'	−298/−287
−262/−254	5'-GACTTAATT-3'	−260/−254
−219/−206	5'-AAATTTAATTTgTA-3'	−218/−206

This study examined approximately –2 kb upstream of promoter. LHX2 binding sites are based on the results of Kato *et al.* [14].

Transcriptional activity of porcine Cga promoter by LHX2 and LHX3

In LβT2 cells, LHX2 and LHX3b repressed *Cga* promoter activity over the −101 bp region, while LHX3a and LHX-del repressed activity over the −240 bp region (Fig. 3A). Repression observed in the −239/−101 bp region was stronger by LHX2 compared with LHX3s. In contrast, in CHO cells, although LHX2 and LHX3a were slightly activated in the −100/+12 bp region, further activation with LHX2 and LHX3a was observed over the −240 bp region. These results indicate that activation occurred between −551/−101 bp.

Transcriptional activity of porcine Fshβ promoter by LHX2 and LHX3

LHX2 and LHX3b showed repressive activity for the *Fshβ* promoter in LβT2 cells (Fig. 3B), as shown for the *Cga* promoter. LHX2 showed notable repression of FSHβ (−103/+10), which showed putative binding site for LHX2, and more intense repression from −104 to −1965 bp. LHX3s showed rather weak repression from −104 to −1965 bp. In contrast to the activity in LβT2 cells, LHX2 and LHX3s activated the *Fshβ* promoter. In CHO cells, LHX2 activated for over −239 bp, while LHX3s showed similar activation at −986 bp (Fig. 3B). The results indicate that activation occurred at the −985/−239 bp region for LHX2 and the −1965/−986 bp region for LHX3a.

Lhx3 expression during porcine pituitary development

We previously reported a gradual increase in *Lhx3*-expression during fetal development by RT-PCR [25], which was similar to that of *Lhx2*-expression [14]. Real-time PCR during porcine pituitary development revealed that the expression of both isoform genes gradually increased before birth, followed by an appreciable decrease by P230 (Fig. 4). The expression level of *Lhx3a* was approximately 20–100-fold higher than that of *Lhx3b* in both sexes, and the expression level of *Lhx3a* and *Lhx3b* was higher in females than in males.

Immunohistochemistry of LHX2 and LHX3

Immunohistochemistry of LHX2 and LHX3 in the postnatal pituitary (P15) was performed using specific antibodies. LHX2-positive cells were not observed in the anterior lobe of the embryonic pituitary. However, a small number of LHX2-positive cells were observed in the postnatal pituitaries, a part of which was the gonadotrope (Fig. 5A), and they were abundant in the posterior lobe but not in the intermediate lobe. In contrast, LHX3-positive cells were observed in the anterior and intermediate lobes, but not in the posterior lobe (data not shown). Double staining with antibodies for hormones and S100β, a non-endocrine cell marker, showed that LHX3 was expressed in most FSHβ-cells as well as in most TSHβ-, PRL-, and S100β-positive cells, but not in GH- and ACTH-positive cells (Fig. 5B).

Discussion

LHX2 and LHX3 belong to the LIM-homeobox family and are composed of two LIM domains (protein-protein interaction) and a homeobox domain (DNA binding domain). Cumulative data have shown that two related factors modulate the gene expression of porcine *Fshβ* [10, 14] and *Cga* [8, 35, 36] and sharing their targets. However, the similarity of their amino acid sequences is quite low (approximately 50% similarity in the LIM and homeobox domains, as shown in Fig. 1), raising the question as to whether these proteins have similar functions. Thus, we compared LHX2 and LHX3 activity in the regulation of *Cga* and *Fshβ*. Binding and reporter assays demonstrated that LHX2 and LHX3 share similar DNA regions but show differences in binding specificity and transcription activity. However, immunohistochemistry showed that LHX2, but not LHX3, was present in a small number of gonadotropes.

The gel mobility shift assay and DNase I footprinting of LHX2 and LHX3 suggested differences in binding specificities. Tables 1 and 2 summarize the binding sites of LHX2 and LHX3 at the promoter region of *Cga* and *Fshβ*. Most sites contained the required core motif, TAAT/ATTA, for homeobox-type transcription factors [16–19]. We previously verified using the SELEX method that LHX2 and LHX3 showed binding affinity for the hexa-nucleotides TAATTA and $^{T}/_{A}{}^{A}/_{T}{}^{T}/_{A}T^{A}/_{T}A$, respectively [14], and discussed that dissimilarity may have been caused by amino acid differences in the homeodomain, a DNA binding domain composed of 60 amino acids making up three α-helixes [21, 22] (Fig. 6). Two proteins showed only 43% similarity in the homeodomain, with differences in 4 of the 14 amino acids in the essential DNA binding sites, including

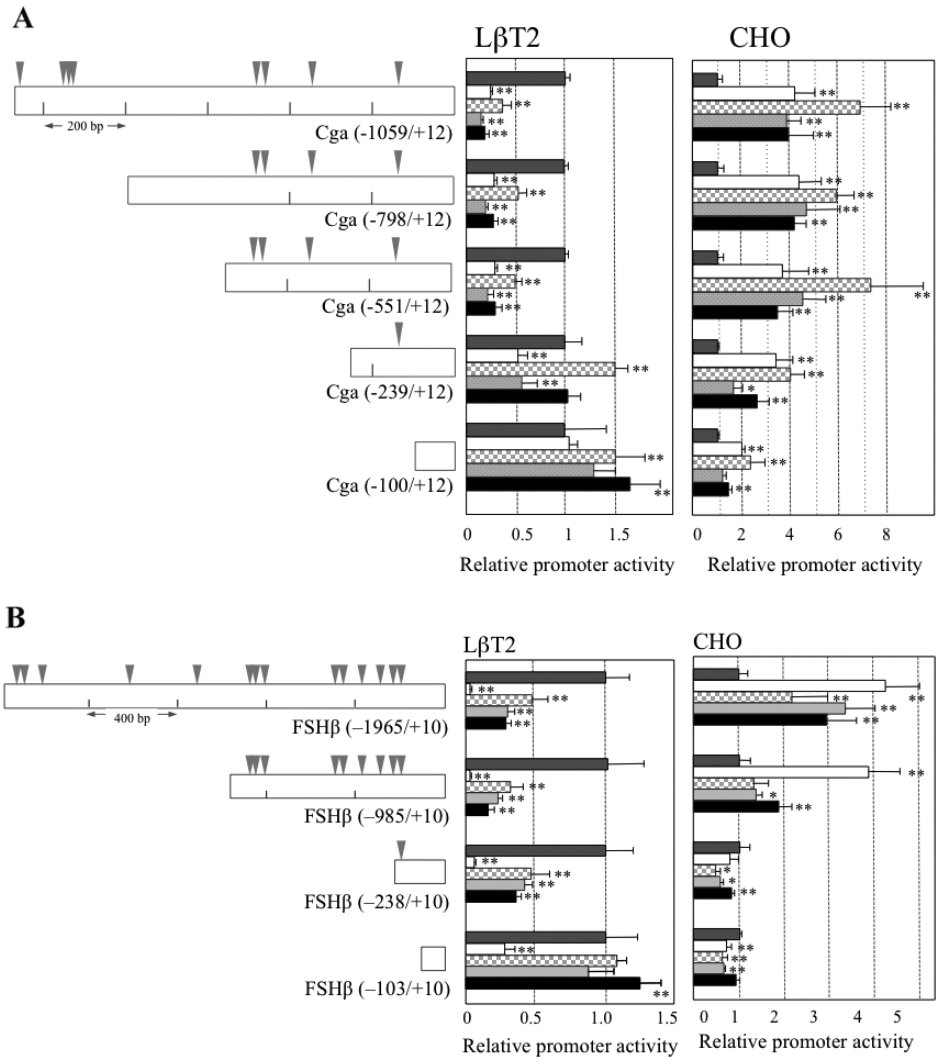

Fig. 3. Transient transfection assay of porcine *Cga* and *Fshβ* promoters in LβT2 and CHO cells. Diagram of the truncated fragments of *Cga* (A) and *Fshβ* (B) promoters fused with the SEAP gene in the pSEAP2-Basic vector are indicated in the left panel. Inverted triangles represent the binding site common (gray) to LHX2 and LHX3. Transfection was performed in LβT2 (middle panel) and CHO (right panel) cells with pcDNA3.1 harboring without (dark-gray bar), with *Lhx2* (open bar), and *Lhx3a* (checked bar), *Lhx3b* (light-gray bar), or *Lhx3-del* (solid bar). An aliquot of cultured medium was used for the SEAP assay. Reporter gene activities are indicated relative to that of the pcDNA3.1 vector. Data (mean ± SD) are the means of quadruplicate transfections from two independent experiments. Asterisks indicate statistical significance by Dunnett's test with F-test. * $P < 0.05$, ** $P < 0.01$.

position 2 (Arg and Lys), position 24 (Asn and Lys), position 31 (Leu and Arg), position 42 (Lys and Met), and position 57 (Arg and Lys), providing variations in polarity, charge, and steric configuration. While Wilson *et al*. [22] demonstrated that residues at 28 (Ile) and 43 (Ala), which are among the five amino acids fundamental for dimerization, are important for hydrophobic interactions, LHX2 and LHX3 contained different amino acids, indicating that they do not form dimers through the homeodomain, as previously observed for paired related homeobox 2 [37].

The variance in transcriptional activities between LHX2 and LHX3s depend on the cell type (Fig. 3), indicating the presence of cell type-specific interactors with both factors to modify their activities in addition to differences in the transcriptional ability. Indeed, the LIM domain of LHX2 and LHX3 are well-known to interact with different classes of transcription factors and/or co-factors [15, 38]. These complexes are involved in diverse biological functions. Various proteins, including CLIM2/NL1/LDB1 and ISL1 [39], PIT1 [40], SF1 [41], and SLB [41], have been reported to interact with LHX3. Our cumulative research demonstrated that CLIM2, a co-factor, is capable of binding to LHX2 and that the interaction with CLIM2 represses LHX2-dependent stimulation of *Cga* expression [36]. Furthermore, CLIM2 is known to interact with several types of LIM-only proteins [42], such as single-stranded binding protein 2 (SSBP2) [43], in the pituitary to form a large complex with various transcription factors

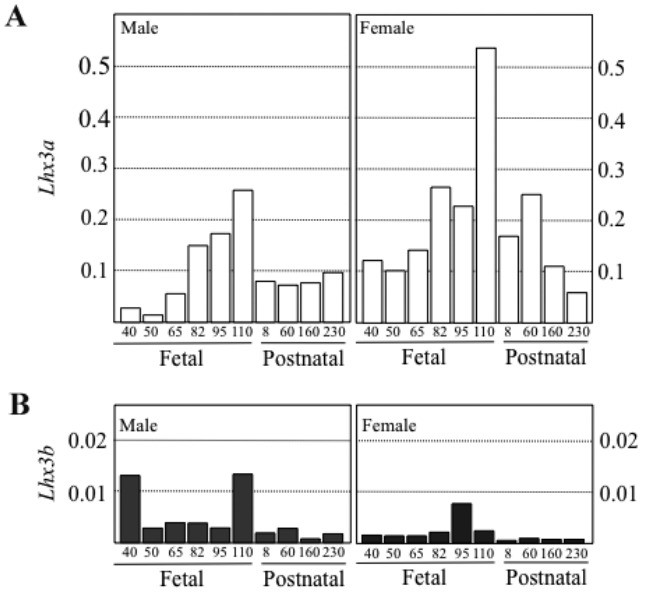

Fig. 4. Real-time PCR analysis of *Lhx3* mRNAs in porcine anterior pituitaries. *Lhx3a* (A) and *Lhx3b* (B) mRNAs of male and female porcine anterior pituitaries were quantified by real-time PCR, and the results are indicated as the relative amounts of *Lhx3a* and *Lhx3b* compared to *cyclophilin A*. Numbers indicated at horizontal axis indicate fetal and postnatal days.

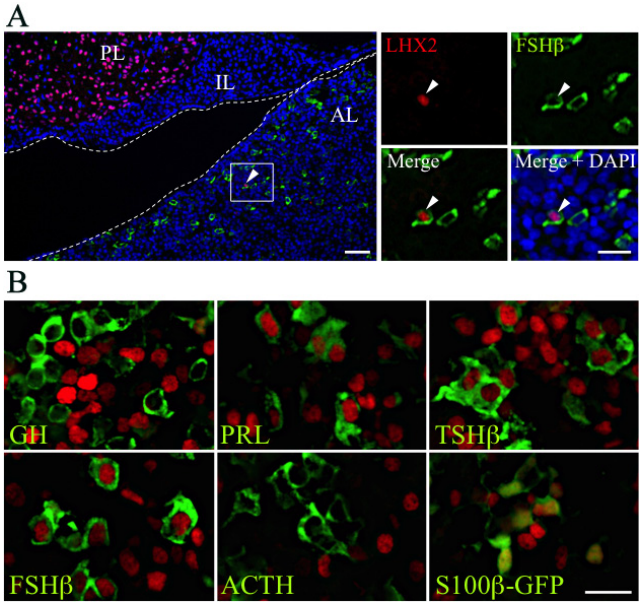

Fig. 5. Immunohistochemistry of LHX2 and LHX3 in the postnatal pituitary. (A) Double-immunostaining of LHX2 (Cy3, red) and FSHβ (Cy5, green) for section on P15 was performed. Merged image with DAPI (nuclei) is shown in the left panel and boxed areas are enlarged in the right panels. Arrowheads indicate LHX2/FSHβ-double positive cells. Dotted lines indicate Rathke's cleft. AL, anterior lobe; IL, posterior lobe; PL, posterior lobe. Bars in the left and right panels indicate 50 and 20 μm, respectively. (B) Double-immunostaining of LHX3 (Cy3, red) and pituitary hormones (Cy5, green with false color) or S100β-GFP (FITC, green) for section on P15 were performed. Bar indicates 20 μm.

(TAL1, E47, and GATA-1) and with LMO2 [44–46] in other tissues. Additionally, it was reported that LHX2 can recruit a co-activator p300, a TATA-binding protein, through the LHX2-binding protein MRG1 [35]. These various interacting partners make it possible to respond to the diverse demands of gene regulation.

The immunohistochemistry results for LHX2 were unexpected. This factor was first cloned from the pituitary gonadotrope lineage cell line αT3-1 [12] and from the porcine anterior pituitary using a Yeast Two-Hybrid System [14]. In the latter, the LHX2 cDNA clone was obtained through repeated screenings and the LHX3 cDNA clone was never found, indicating a stronger DNA binding ability for LHX2 than for LHX3. Zhao *et al*. described the absence or barely detectable level of *Lhx2* in the embryonic anterior lobe without a description of the postnatal tissue by *in situ* hybridization [12]. Collectively, LHX2 may be transiently expressed in the gonadotrope as well as in other pituitary cells and regulate *Fshβ* and *Cga* by competing with LHX3. In contrast, immunohistochemistry for LHX3 demonstrated that this factor resides in select pituitary cells, including FSHβ-, PRL-, TSHβ-, and S100β-positive cells, in the postnatal adult anterior lobe. How *Lhx3* discriminates between

position / factor	1	helix-1 10	22	helix-2 28	37	helix-3 42	60
HESX1	GRRPRTAFT	QNQIEVLENVFRV	NCYPG	IDIREDLAQK	LNLE	EDRIQIWFQNRRAKLKRSH	
PROP1	RRRHRTTFS	PAQLEQLESAFGR	NQYPD	IWAREGLARD	TGLS	EARIQVWFQNRRAKQRKQE	
Paired Q50	QRRSRTTFS	ASQLDELERAFER	TQYPD	IYTREELAQR	TNLT	EARIQVWFQNRRARLRKQH	
PRRX2	QRRNRTTFN	SSQLQALERVFER	THYPD	AFVREELARR	VNLS	EARVQVWFQNRRAKFRRNE	
LHX2	TKRMRTSFK	HHQLRTMKSYFAI	NHNPD	AKDLKQLAQK	TGLT	KRVLQVWFQNARAKFRRNL	
LHX3	AKRPRTTIT	AKQLETLKSAYNT	SPKPA	RHVREQLSSE	TGLD	MRVVQVWFQNRRAKEKRLK	
	* 3			* 28		* * 43 45	* 55

Fig. 6. Comparison of the amino acid sequences of the homeodomains. Amino acid sequences of homeodomain of HESX1 (Accession number: NM_010420) [21, 22], PROP1 (Accession number: AB187272) [23], paired Q50 (Accession number: 1FJL_A) [20], and PRX2 (Accession number: D00584745) [26] are compared with those of porcine LHX2 and LHX3. Amino acid residues important for dimerization and for DNA-binding are marked with an asterisk (*) and shaded, respectively.

cell types remains to be clarified.

In summary, we demonstrated that LHX2 and LHX3s differ in their DNA binding specificities and transcriptional activities. In addition, these proteins showed cell type-dependent activity. The population of LHX2-positive cells was very small, while LHX3s collectively resided in gonadotrope cells. It would be interesting to evaluate the cross-talk between LHX2 and LHX3s in cells expressing both proteins.

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