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Regular Article

Expression of Retinaldehyde Dehydrogenases in the Pituitary Glands of Fetus and Adult Mice

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Retinoic acid (RA) plays a critical role in cell growth and tissue development. RA is synthesized from retinoids through oxidation processes by the retinaldehyde dehydrogenase (Raldh) family. However, the expression of *Raldhs* during pituitary development and the identification of *Raldh*-expressing cells in the adult pituitary have not been fully elucidated. Here, we performed *in situ* hybridization to localize the three Raldh isoforms (*Raldh1-3*) in fetal and adult mouse pituitary glands. The results showed that *Raldh2* expression was observed in Rathke's pouch from embryonic day 13.5 (E13.5), and this expression was sustained in the anterior lobe of the pituitary primordium from E15.5 to E17.5. In contrast, *Raldh1* and *Raldh3* were rarely detectable. Real-time PCR analysis revealed that *Raldh2* was the predominant isoform expressed in the adult pituitary, although *Raldh1* was also expressed to a lesser extent. In the adult pituitary, *Raldh1*-expressing cells were primarily observed in the posterior lobe. *Raldh2*-expressing cells were found in the marginal cell layer and parenchyma of the anterior lobe and were immunopositive for aldolase C (folliculostellate cells), but not for anterior pituitary hormones. These results suggest that RA is an important regulatory factor in the functions of the pituitary throughout its development in mice.

Key words: mouse, retinoic acid, anterior pituitary gland, Raldh

I. Introduction

Retinoic acid (RA) is a lipid-soluble hormone that is an essential signaling molecule for normal embryonic development and tissue differentiation in vertebrates. RA binds to two families of intracellular retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which have DNA binding domain and directly regulate gene expressions after receiving RA. RARs and RXRs are expressed in both embryonic [8, 9] and adult anterior pituitary glands [23, 33]. RA is thought to be one of the regulatory molecules for pituitary development and pituitary functions.

RA is converted from retinal by retinaldehyde dehydrogenases (Raldhs). Raldh1 (gene: Aldh1a1), Raldh2 (gene: Aldh1a2), and Raldh3 (gene: Aldh1a3) catalyze the oxidation of retinal to RA with different substrate specificities [for a review, see Ref. 10]. We have previously reported that *Raldh2* and *Raldh3*, but not *Raldh1*, are highly expressed in the embryonic anterior pituitary gland of the rats [12]. On the other hand, in the adult rat pituitary gland, *Raldh1* is predominantly expressed among *Raldhs* [11]. Thus, we demonstrated that expression of Raldh isoforms can be altered during pituitary development. In mice, *Raldh2* was found to be expressed in developing pituitary [26], however, the expression of other isoforms, alteration of isoform during development, and characterization of

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Raldh-expressing cells have not been elucidated. The present study examined the expression profile of *Raldh1*, *Raldh2*, and *Raldh3* during mouse pituitary development, and identified *Raldh*-expressing cells in adult mouse pituitary gland.

II. Materials and Methods

Animals

Adult C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained on a 12-hr light/dark cycle and given conventional food and water *ad libitum*. The room temperature was controlled at around 22°C. The day on which spermatozoa were found in a vaginal smear was designated as embryonic day 0.5 (E0.5). All animals were performed after receiving approval from the Institutional Animal Experiment Committee of Kanagawa University and were conducted in accordance with the Institutional Regulations for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science and Technology of Japan.

in situ hybridization and immunohistochemistry

Mice were sacrificed via exsanguination from the right atrium under deep anesthesia with isoflurane (Viatris Inc., Tokyo, Japan). The embryos and pituitary tissues were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20–24 hr at 4°C. The tissues were immersed for more than 2 days in cacodylate buffer containing 30% sucrose at 4°C. Tissues were then embedded in Tissue-Tek compound (Sakura Finetek, Tokyo, Japan) and frozen rapidly. A cryostat (CM3000; Leica Microsystems, Wetzlar, Germany) was used to make sections (thickness, 8 μm; sagittal sections for prenatal samples and frontal sections for postnatal samples), which were then mounted on MAS-coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan).

in situ hybridization was performed as described in our previous reports [12, 29]. *Raldhs* cDNA fragments were amplified from mouse pituitary gland cDNA by PCR using gene-specific primers (Table 1). Amplified cDNA fragments were ligated into pGEM-T easy vector (Promega, Madison, WI, USA) and cloned. Gene-specific antisense or sense digoxigenin (DIG)-labeled cRNA probes were made using the Roche DIG RNA labeling kit (Roche Diagnostics GmbH, Penzberg, Germany). DIG-labeled cRNA probe hybridization was performed in solution containing 50% formamide, 10% dextran sulfate (Wako Pure

Table 1. *List of primers used for PCR*

Synonym	Gene	GenBank accession number	Primer sequence (5'-3')	Product size (bp)	Application
Raldh1	Aldh1a1	NM_013467.1	Forward: TTATCATCAAGGCCAATGCT Reverse: CACCCAGTTCTCTTCCATTT	551	ISH
			Forward: TCCTCTGACCCCAGGAATAA Reverse: CACACTCCAGTTTGGCTCCT	110	Real-time PCR
Raldh2	Aldh1a2	NM_009022.4	Forward: ATGGGTGAGTTTGGCTTACG Reverse: CCTGCTGGAAGGACTCAAAG	562	ISH
			Forward: TTGCCTCACAACAAGTGAGC Reverse: ACCAAATGGGGTTCATTGGA	112	Real-time PCR
Raldh3	Aldh1a3	BC058277.1	Forward: CGACCTGGAAGGCTGTATTA Reverse: CTCTTCTTGGCGAACTCCAC	627	ISH
			Forward: TCATCAAAGAGGTCGGGTTC Reverse: GCTTTCCAACCTCTGTGGAG	135	Real-time PCR
Gapdh	Gapdh	NM_008084.2	Forward: AAGGGCTCATGACCACAGTC Reverse: GGATGCAGGGATGATGTTCT	116	Real-time PCR

ISH: *in situ* hybridization

Table 2. *Primary antibodies*

Antigen	Immunized animal	Dilution factor	Supplier
mouse Aldo C	rabbit	1 μg/ml	Frontier Institute Co., Ltd., Hokkaido, Japan
rat GH	rabbit	1:10,000	Dr. K. Wakabayashi, Gunmma University, Japan
rat PRL	rabbit	1:10,000	Dr. K. Wakabayashi, Gunmma University, Japan
ovine LH β-subunit	rabbit	1:5,000	Dr. K. Wakabayashi, Gunmma University, Japan
porcine ACTH 1-39	rabbit	1:10,000	Dr. H. Nakamura, Hokkaido University, Japan
rat TSHβ-subunit	guinea pig	1:60,000	Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Disease

Aldo C, Aldolase C; GH, growth hormone; PRL, prolactin; LH, luteinizing hormone; ACTH, adrenocorticotrophic hormone; TSH, thyroid stimulating hormone

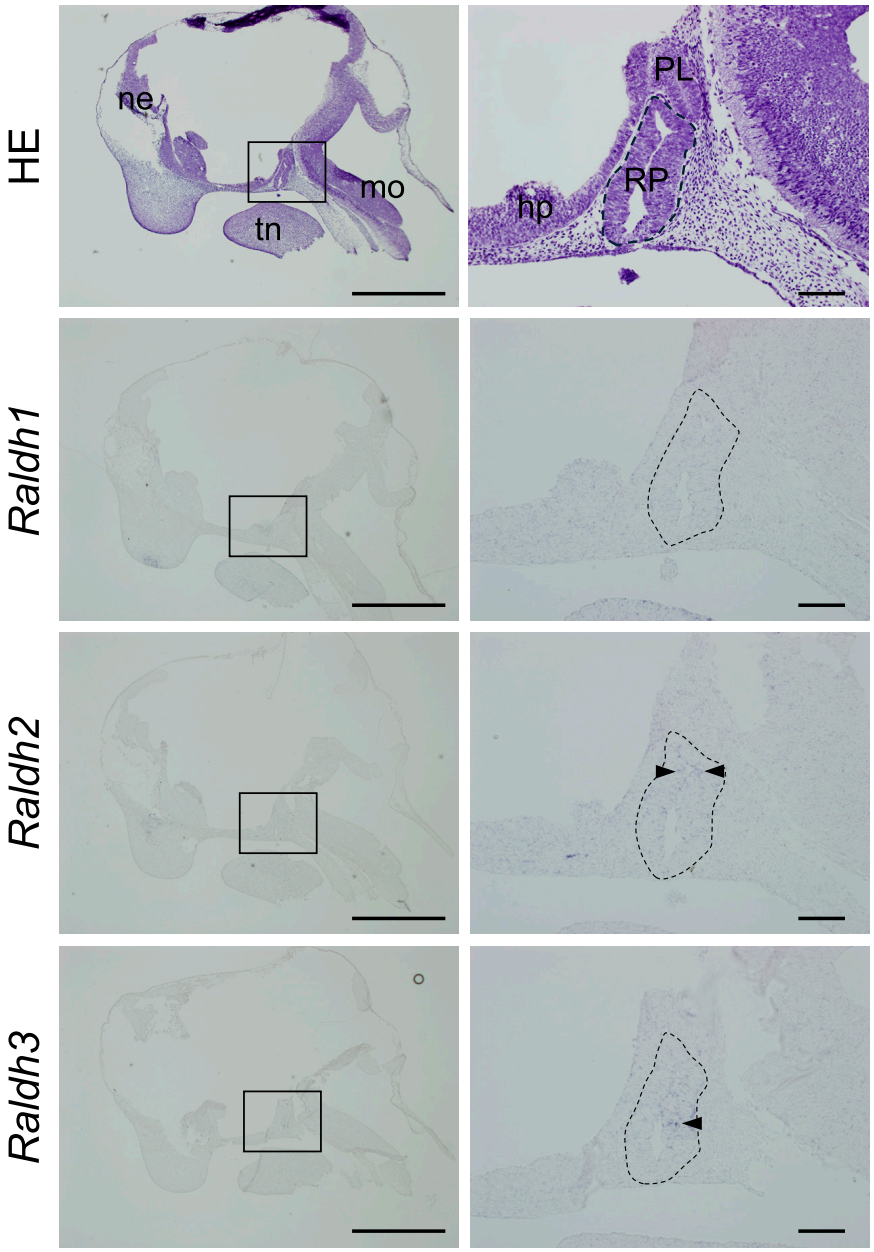

Fig. 1. *In situ* hybridization of *Raldh1*, *Raldh2*, and *Raldh3* at embryonic day 11.5 (E11.5), sagittal view (rostral part faces to the left). Right panel is a high-magnification view of the Rathke's pouch (RP; dotted line) in the left panel. In the mouse E11.5 embryo, a formation of the RP is observed (HE; hematoxylin-eosin stain). *Raldh2* and *Raldh3* mRNA are rarely detected in the RP (arrowheads). hp; hypothalamus, mo; medulla oblongata, ne; neopallia cortex, PL; posterior lobe, tn; tongue. Bars = (left panel) 1 mm; (right panel) 100 µm.

Chemical Ind., Ltd., Osaka, Japan), 3x saline-sodium citrate, 120 mM phosphate buffer (pH 7.4), 1x Denhardt's solution (Nacalai Tesque Inc., Kyoto, Japan), 125 µg/ml tRNA (Life Technologies, Carlsbad, CA, USA) and 100 µg/ml sonicated sperm DNA (Life Technologies) at 55°C for 16–18 hr. Visualization of each type of mRNA was performed with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics GmbH) using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics GmbH). A control experiment was performed, and no specific signal was detected in a section processed with DIG-labeled sense RNA probe.

For double-staining, after *Raldh2* mRNA had been detected by *in situ* hybridization, the section was immunostained as described in our previous reports [1, 11] After *in situ* hybridization, sections were incubated in phosphate buffered saline (PBS) containing 2% normal goat serum for 30 min. They were incubated in PBS with primary antibodies for overnight at around 22°C. The primary antibodies are shown in Table 2, as reported previously [16]. After washing with PBS, sections were incubated in PBS with

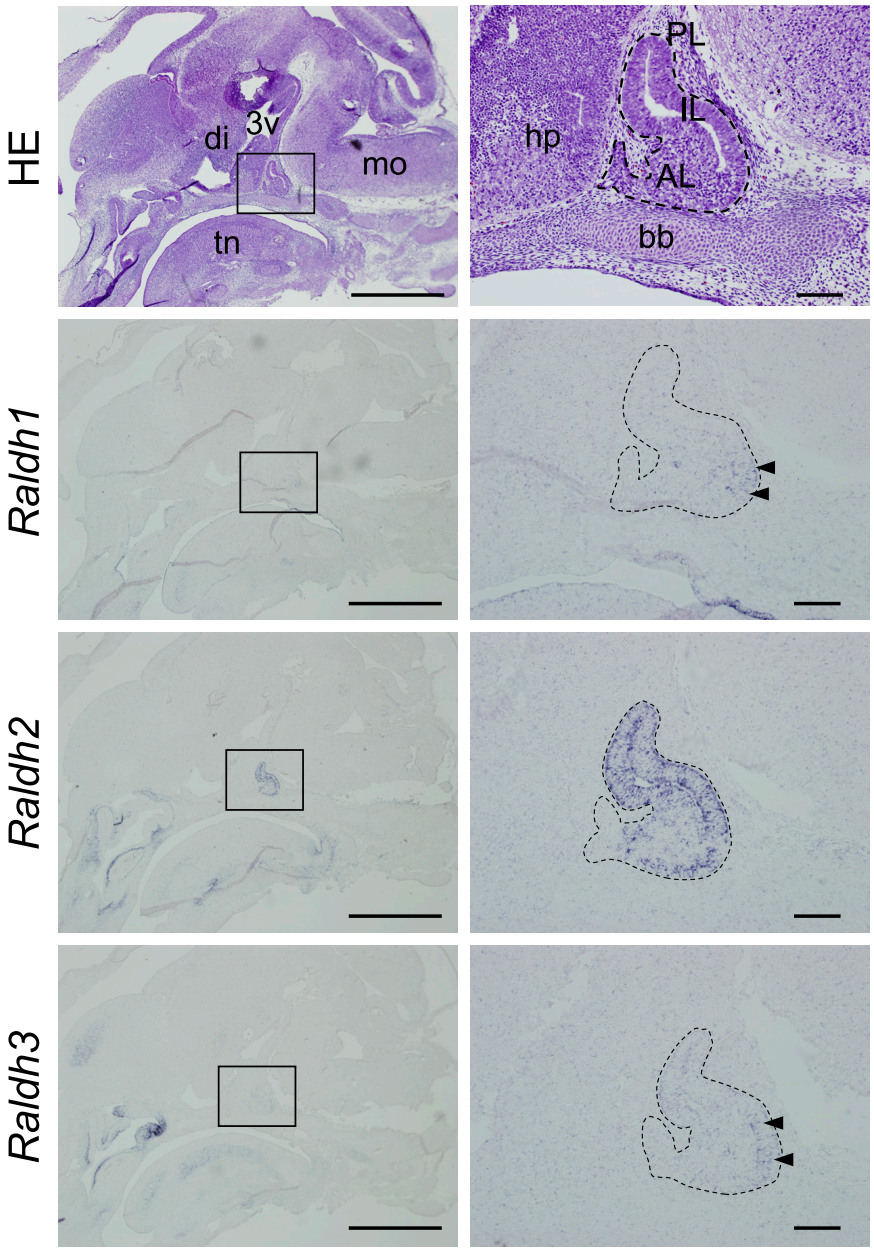

Fig. 2. *In situ* hybridization of *Raldh1*, *Raldh2*, and *Raldh3* at E13.5, sagittal view (rostral part faces to the left). Right panel is a high-magnification view of the pituitary primordium (dotted line) in the left panel. In the mouse E13.5 embryo, parts of anterior lobe (AL) and intermediate lobe (IL) are formed (HE; hematoxylin-eosin stain). *Raldh2* mRNA is markedly detected in the AL and IL. *Raldh1* and *Raldh3* mRNA are faintly detected in the AL and IL (arrowheads). 3v; third ventricle, bb; basisphenoid bone, di; diencephalon, hp; hypothalamus, mo; medulla oblongata, PL; posterior lobe, tn; tongue. Bars = (left panel) 1 mm; (right panel) 100 μm.

biotinylated goat IgG anti-rabbit IgG or biotinylated goat IgG anti-guinea pig IgG (Vector Laboratories, California, USA) for 30 min at around 22°C. The ABC method (Vector Laboratories) was performed with 3,3'-diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan) as substrate.

Quantification of mRNA levels by real-time PCR

Total RNA fractions were prepared with RNeasy Mini Kit (Qiagen, Hilden, Germany) from pituitary tissues according to the manufacturer's instructions. They were incubated with RNase-free DNase Kit (Qiagen). Quantitative real-time PCR was performed as described in our previous reports [18, 22]. cDNA was synthesized by a PrimeScript First Strand cDNA Synthesis Kit with oligo-$(dT)_{20}$ primer (Takara Bio, Otsu, Japan). Quantitative real-time PCR (ABI PRISM 7900HT; Applied Biosystems, Foster City, CA USA) was performed using gene-specific primers and SYBR Premix Ex Taq (Takara Bio) containing SYBR Green I. The sequences of the gene-specific primers

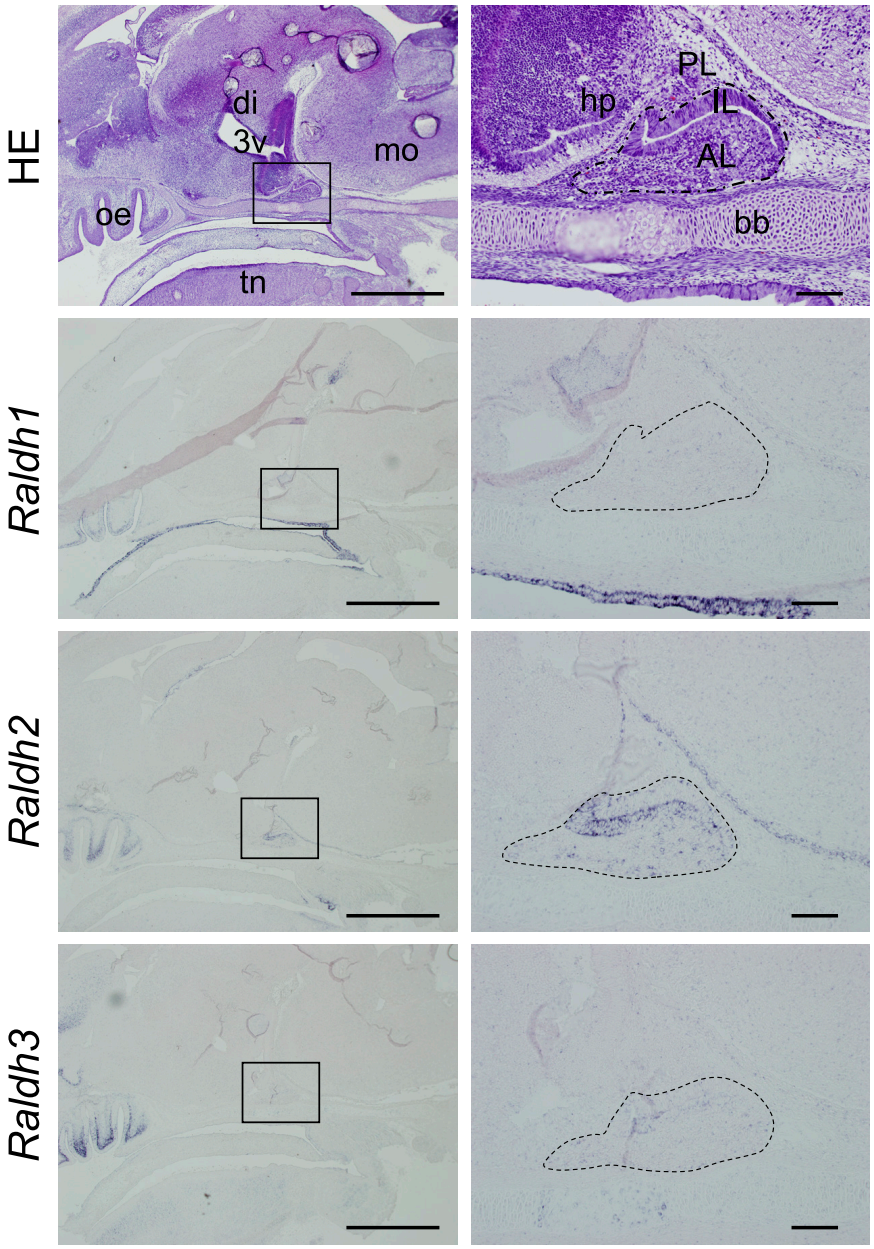

Fig. 3. *In situ* hybridization of *Raldh1*, *Raldh2*, and *Raldh3* at E15.5, sagittal view (rostral part faces to the left). Right panel is a high-magnification view of the pituitary primordium (dotted line) in the left panel. *Raldh2* mRNA is strongly detected in the anterior lobe (AL), but not in the intermediate lobe (IL). 3v; third ventricle, bb; basisphenoid bone, di; diencephalon, hp; hypothalamus, mo; medulla oblongata, oe; olfactory epithelium, PL; posterior lobe, tn; tongue. Bars = (left panel) 1 mm; (right panel) 100 μm.

are listed in Table 1. For normalization, we also quantified glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The relative gene expression was calculated by comparing the cycle times for each target PCR. Cycle threshold values were converted to relative gene expression levels by using the $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$ method.

Statistical analysis

The data were presented as means ± SEM for three animals. The Dunnett test was used for statistical analysis. Differences between genes were considered statistically significant when the *P* value was less than 0.01.

III. Results

Localization of Raldh mRNAs in mouse embryonic pituitary gland

At embryonic day 11.5 (E11.5), Rathke's pouch has been formed from oral ectoderm. *Raldh2* and *Raldh3* were rarely expressed in the Rathke's pouch (Fig. 1). At E13.5,

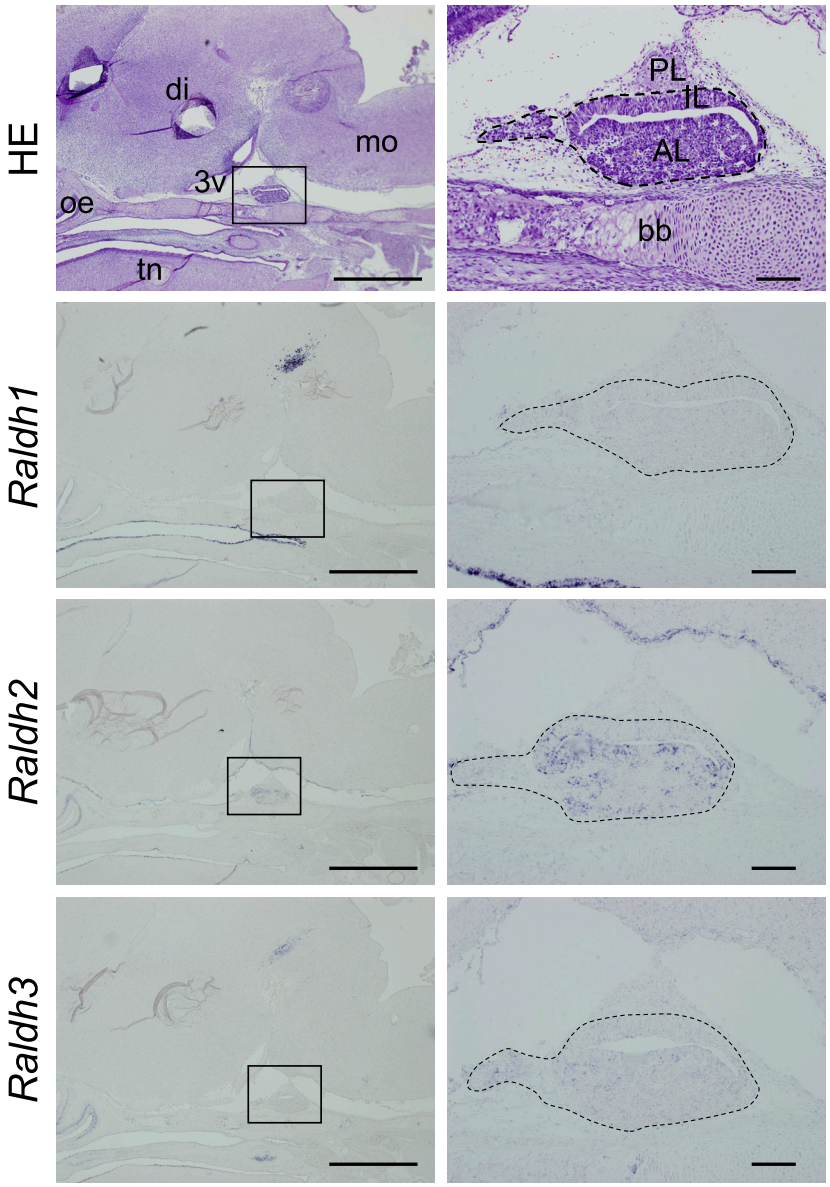

Fig. 4. *In situ* hybridization of *Raldh1*, *Raldh2*, and *Raldh3* at E17.5, sagittal view (rostral part faces to the left). Right panel is a high-magnification view of the pituitary primordium (dotted line) in the left panel. *Raldh2* mRNA remains detected in the anterior lobe (AL). 3v; third ventricle, bb; basisphenoid bone, di; diencephalon, IL; intermediate lobe, PL; posterior lobe, mo; medulla oblongata, oe; olfactory epithelium, tn; tongue. Bars = (left panel) 1 mm; (right panel) 100 μm.

the primordium of anterior and intermediate lobes of pituitary gland has been formed (Fig. 2). *Raldh2* was strongly expressed in the pituitary primordium, while *Raldh1* and *Raldh3* mRNAs were faintly detectable (Fig. 2). E15.5–E17.5, *Raldh2* expression was dominant, and the localization was persisted in the primordium of anterior lobe (Fig. 3, Fig. 4), while *Raldh1* and *Raldh3* expressions were modest.

Quantitative analysis of Raldh expressions in adult mouse pituitary gland

Raldh1, *Raldh2*, and *Raldh3* expressions in whole pituitary gland from 8-week-old male mice were quantified by real-time PCR (Fig. 5). *Raldh2* expression was the highest among 3 *Raldhs*, and *Raldh1* was expressed to a lesser extent. However, *Raldh3* expression was significantly low when compared with *Raldh1* and *Raldh2*.

Raldh-expressing cells in the adult mouse pituitary gland

Raldh1 mRNA was detected in the adult mouse pituitary gland by *in situ* hybridization using a DIG-labeled antisense cRNA probe (Fig. 6). *Raldh1*-expressing cells were found mostly in the posterior lobe (Fig. 6C), but a few in the marginal cell layer and parenchyma of anterior lobe (Fig. 6C, D). On the other hand, *Raldh2*-expressing cells were found specifically in the anterior lobe (Fig. 7B).

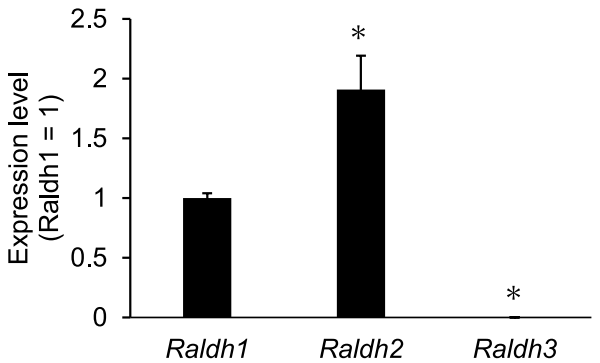

Fig. 5. *Raldhs* expression levels in the adult male pituitary gland determined by real-time PCR. The expressions of *Raldh1*, *Raldh2* and *Raldh3* mRNA were normalized with an internal control (*Gapdh*) (mean ± SEM, n = 3). Relative *Raldh2* and *Raldh3* mRNA levels against *Raldh1* values are shown. * $P < 0.01$ *vs. Raldh1* mRNA level.

Raldh2-expressing cells were located in the marginal layer that lines the Rathke's cleft (Fig. 7C), and cluster of *Raldh2*-expressing cells were scattered in the parenchymal region of the anterior lobe (Fig. 7D).

Characterization of Raldh2-expressing cells in mouse anterior lobe

The expression of *Raldh2* in five types of hormone-producing cells and folliculostellate (FS) cells was examined by double staining of *in situ* hybridization and immunohistochemistry. *Raldh2* mRNA was not found in the cells producing adrenocorticotrophic hormone, growth hormone (GH), prolactin (PRL), thyroid stimulating hormone (TSH), or luteinizing hormone (Fig. 8A–E). Aldolase C is a novel marker molecule for rodent FS cells [16]. *Raldh2* mRNA was only expressed in some FS cells which was identified by aldolase C immunoreactivity (Fig. 8F) in the anterior pituitary gland.

IV. Discussion

The present study demonstrated that *Raldh2* was strongly expressed in the pituitary primordium of mouse embryos. This finding is consistent with a previous report where *Raldh2* expression was observed from E12.5 of mouse pituitary gland [26]. In contrast, in rat embryo, *Raldh3* as well as *Raldh2* were strongly expressed in the pituitary primordium [12]. In avian and teleost, *Raldh3* was expressed in their developing pituitary [17, 21, 28, 32]. These reports suggest that RA is synthesized during pituitary development across a wide range of vertebrates, although there are species differences in the isoforms of *Raldh* expressed. Indeed, our previous report showed that RA increases the expression level of the pituitary-specific transcription factor Prop1 in the rat Rathke's pouch [34]. Other groups also demonstrated using rats that RA stimulates the expression of Pit-1 (Pou1f1), a transcription factor essential for differentiation toward GH, PRL, and TSH-producing cells in the anterior pituitary glands [31], and that RA induces GH-producing cell differentiation *in vitro* [5, 24]. In sum, RA is suggested to act as an important signaling molecule for pituitary organogenesis.

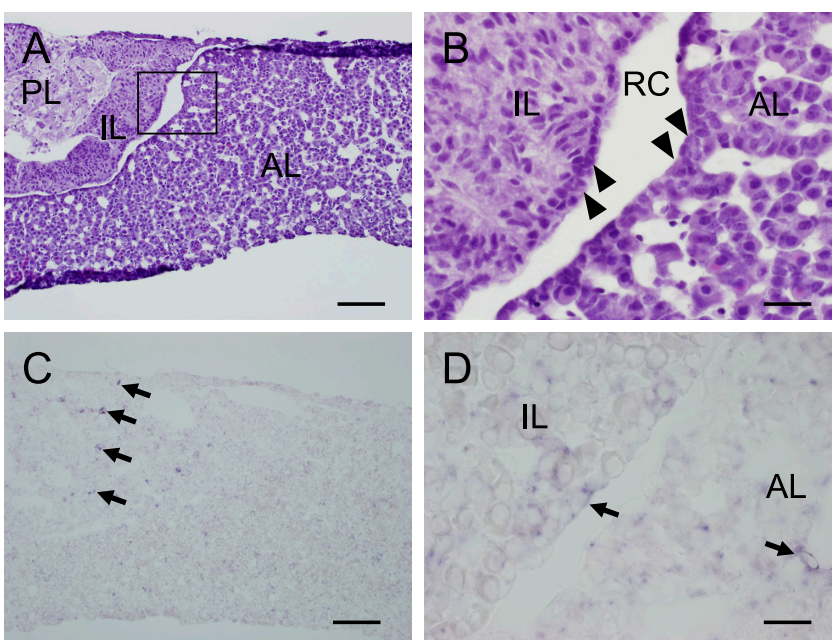

Fig. 6. *In situ* hybridization for *Raldh1* mRNA in the adult mouse pituitary gland. **A**, **B** Hematoxylin and eosin staining of a frontal section of the pituitary gland (AL; anterior lobe, IL; intermediate lobe, PL; posterior lobe, RC; Rathke's cleft, arrowheads; marginal cell layer). Magnified views of the rectangular areas in **A** are shown in **B**. *Raldh1*–expressing cells (arrows) are detected in the PL (**C**). *Raldh1*–expressing cells are barely observed in the marginal cell layer and in the AL (**D**). Bars = (**A, C**) 100 μm; (**B, D**) 20 μm.

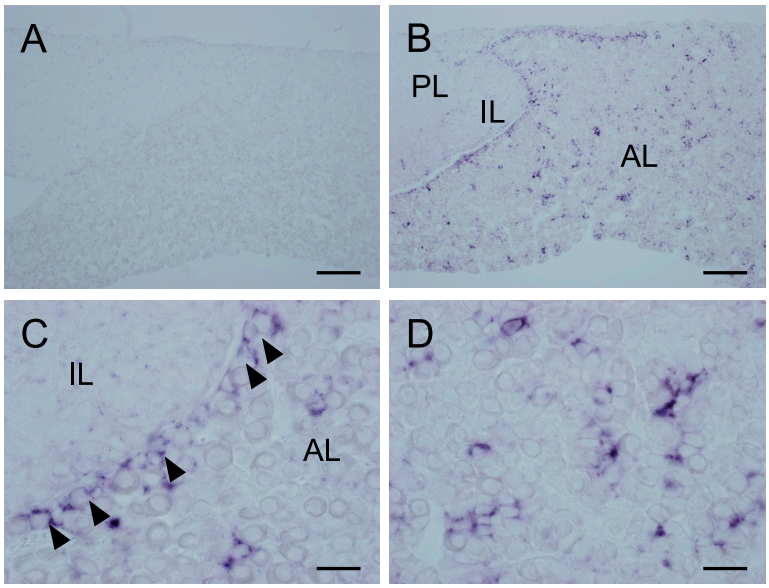

Fig. 7. *In situ* hybridization for *Raldh2* mRNA in the adult mouse pituitary gland. *Raldh2*–expressing cells are observed in the anterior lobe (AL), but not in the intermediate lobe (IL) and posterior lobe (PL). *Raldh2*–expressing cells are located in the marginal cell layer (arrowheads in **C**) and scattered or aggregated in the parenchyma of anterior lobe (**D**). No specific signal is observed when the section was processed with a DIG-labeled sense RNA probe (**A**). Bars = (**A, B**) 100 μm; (**C, D**) 20 μm.

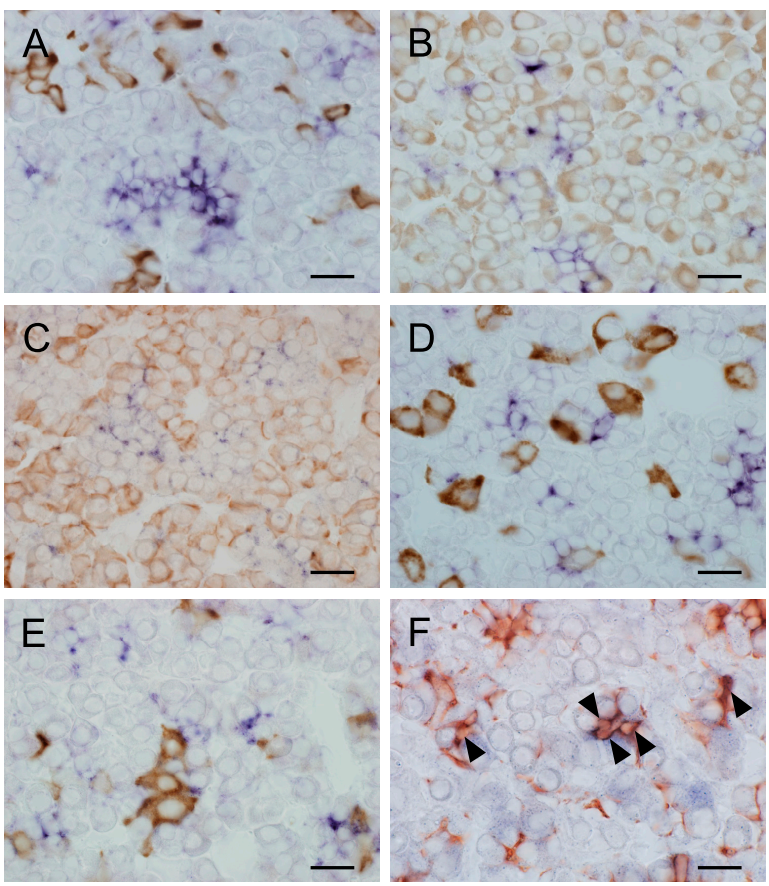

Fig. 8. Double-staining of *Raldh2* detected by *in situ* hybridization, and pituitary hormones or aldolase C detected by immunohistochemistry, in adult anterior pituitary gland. *Raldh2* mRNA was undetectable in anterior pituitary cells expressing ACTH (**A**), GH (**B**), prolactin (**C**), TSHβ (**D**), and LHβ (**E**). *Raldh2* mRNA is detected in some aldolase C-positive folliculostellate cells (**F**, arrowheads). *In situ* hybridization with NBT/BCIP signals are shown in purple and immunohistochemistry with DAB in brown. Bars = 20 μm.

The present study first demonstrated that *Raldh2* is expressed in FS cells and the marginal layer cells of the adult mouse pituitary gland. Both cell types are known as major agranular cells in the anterior pituitary gland [7, 16], and our recent studies suggested that they are closely related to each other [19, 20]. In rats, however, *Raldh1* is the major isoform in the adult pituitary, and it is expressed in some populations of prolactin-producing cells, FS cells, and marginal layer cells [11]. Although the isoform of *Raldh* expressed in the mouse and rat pituitary is different, at least FS cells and marginal layer cells could be a major provider of RA in the adult anterior pituitary gland. RA is known to promote many genes such as the type 2 dopamine receptor in mice [30], GH [2, 25], GH-releasing hormone receptor [22, 27], and the ghrelin receptor in rats [22]. Additionally, RA suppresses thyroid-stimulating hormone β-subunit mRNA expression in rats [3, 4]. Our previous studies also showed that the heparin-binding growth factor midkine is expressed in FS cells [15], and RA stimulates midkine expression in rat pituitary [23]. Agranular FS cells and marginal layer cells may control these genes in the anterior pituitary by secreting RA as intracrine/paracrine molecules.

However, the factors controlling the expression of *Raldhs* in pituitary are largely unknown, although there are some reports showing that the transcription factor Prop-1 is thought to regulate *Raldh2* expression during mouse development [6], and that estrogen suppressed the expression of *Raldh1* in the anterior pituitary gland of adult rats [13, 14]. In future studies, it will be necessary to elucidate how the expression of *Raldhs* is temporally and spatially controlled, as this will provide insights into the regulatory mechanisms of RA synthesis at the local level.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Acknowledgments

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