博士論文　（要約）

論文題目　Functional Analysis of Ubiquitin C-Terminal Hydrolase-1 in Pituitary Gland Cells

（下垂体細胞におけるユビキチン C 末端加水分解酵素 1 型（UCH-L1）の機能解析に関する研究）

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General Introduction

Protein degradation is one of essential intracellular activities to maintain normal cellular functions (Weissman, 2001). Firstly, the small 76-amino acid protein ubiquitin can be attached covalently to its substrate proteins. Subsequently, the protein substrate is tagged by a polyubiquitin chain which is recognized by 26S proteasome and result in the degradation (Miller and Gordon, 2005). The degradation of proteins mediated by ubiquitin-proteasome system (UPS) plays important roles in a wide variety of cellular processes, which include intracellular protein degradations, cell cycle control, transcriptional regulation, stress responses as well as apoptosis (Adams, 2003; Ciechanover et al., 1984; Dimmeler et al., 1999). Deubiquitinating enzymes (DUBs) are a group of more than sixty known proteases that regulate ubiquitins by cleaving ubiquitin-protein bonds (Wing, 2003). DUBs are mainly divided into ubiquitin-specific proteases (UBPs) and ubiquitin C-terminal hydrolases (UCHs). UBPs are thought to disassemble polyubiquitin chains, whereas UCHs are considered to catalyze the removal of peptides and small molecules from the C-terminus of ubiquitin (Baarends et al., 2000; Wilkinson, 1997).

Ubiquitin C-terminal hydrolase-L1 (UCH-L1), one of UCHs, was originally isolated and identified as protein gene product (PGP 9.5) (Wilkinson et al., 1989). To date, at least four UCHs isozymes have been identified in mammals (Mayer and Wilkinson, 1989; Wilkinson, 1997). Among them, mouse UCH-L1 and UCH-L3 have 52 % amino acid identity and share significant structural similarity (Kurihara et al., 2001; Osawa et al., 2001; Wilkinson, 1997). UCH-L3 is an ubiquitously expressed protein, whereas the expression of UCH-L1 is mainly restricted to neurons and germ cells (Kent and Clarke, 1991; Schofield et al., 1995; Wilkinson et al., 1989; Wilson et al., 1988). Additionally, these two isozymes

have different enzymatical substrate specificity. Although substrate(s) of these two enzymes *in vivo* has not yet been clearly identified, some studies suggested that UCH-L1 might be efficient to cleave proubiquitin precursor, while UCH-L3 has a preference for ubiquitin ribosomal fusion proteins (Kent and Clarke, 1991; Larsen et al., 1998; Trowern et al., 1996). In addition to these differences, UCH-L1 also possesses dimerization-dependent ubiquitin ligase activity (Liu et al., 2002).

The functional role of UCH-L1 has been widely demonstrated in neurons and germ cells. Reduced level and extensive oxidative modification of UCH-L1 have been observed in the brains of Alzheimer's disease (AD) patients and Parkinson's disease (PD) patients (Castegna et al., 2002; Choi et al., 2004). The I93M mutation in UCH-L1 was identified in a German family affected by dominant inherited PD (Leroy et al., 1998). The loss of dopaminergic (DA) neurons is a hallmark of PD. It has been demonstrated that *Uchl1*^{I93M} transgenic mice showed partial loss of DA, which suggested the involvement of UCH-L1 in the pathogenesis of PD (Setsuie and Wada, 2007). Moreover, gracile axonal dystrophy (*gad*) mice are spontaneous mutants which show degenerative lesion in the most rostral portion of the gracile fascicles by approximately postnatal day 80. Clinical symptoms of affected mice are characterized with ataxia and difficulty in moving the hind limbs. These pathological changes gradually progress and *gad* mice eventually die due to weakness around postnatal day 150. Subsequent study revealed that *gad* mice were autosomal recessive mutant with an intragenic deletion of *Uchl1* gene, and UCH-L1 protein was not detected in this strain of mice. These mutant mice provide a useful model to study the functional role of UCH-L1 (Kikuchi et al., 1990; Saigoh et al., 1999; Yamazaki et al.,

1988b). On the other hand, UCH-L1 has been reported to play a crucial role in germ cells. In *Uchl1* transgenic mice, the overexpression of UCH-L1 induced testicular germ cell apoptosis, resulting in the arrest of spermatogenesis and infertility (Wang et al., 2006). Previous studies on *gad* mice also revealed that UCH-L1 was essential for sperm quality control during spermatogenesis in male mice and UCH-L1 had a function in the plasma membrane of oocytes to block polyspermy in female mice (Kwon et al., 2005; Sekiguchi et al., 2006).

The pituitary gland is a small but important component of the endocrine system. It is composed of three lobes, the anterior lobe, the intermediate lobe and the posterior lobe (Yeung et al., 2006). Among them, the anterior lobe constitutes the major part of the pituitary gland. It is primarily composed of five distinct endocrine hormone-producing cell types based on the trophic hormones which they produce. Corticotropes produce adrenocorticotropic hormone (ACTH), which stimulates the secretion of mineralocorticoid in the adrenal cortex. Somatotropes produce growth hormone (GH), which is responsible for the growth and differentiation of several other tissues. Lactotropes produce prolactin (PRL), which stimulates the milk production in the mammary glands. Thyrotropes produce thyroid-stimulating hormone (TSH), which in turn regulates the activities of the thyroid gland. Gonadotropes produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on gonads. These hormone-producing cells are joined with a non-hormone-producing cell type, the folliculostellate (FS) cells (Inoue et al., 1999; Ooi et al., 2004; Yeung et al., 2006). In general, the composition of hormone-producing cell populations is somatotropes (50 %), lactotropes (10-20 %), thyrotropes (10-20 %), corticotropes (10-

20 %) and gonadotropes (10-20 %), respectively.

Although UCH-L1 has been detected immunohistochemically in the anterior pituitary gland (Kent and Rowe, 1992; Wilson et al., 1988), the understandings of UCH-L1 are largely unknown. In the present study, I attempted to examine the specific expression pattern and the functional role of UCH-L1 in the anterior pituitary gland.

Chapter 1

Ubiquitin C-Terminal Hydrolase L1 is expressed in Mouse Anterior Pituitary Gland

Abstract

The ubiquitin-proteasome system (UPS) plays a fundamental role in regulating various biological activities. UCH-L1 is a deubiquitinating enzyme, belonging to the UPS. To date, it has been reported that UCH-L1 is highly and restrictedly expressed in the neural and reproductive tissues and plays significant roles in these organs. Although the expression of UCH-L1 in the anterior pituitary gland has been reported, the detailed localization and the role of UCH-L1 remain obscure. In this chapter, I detected UCH-L1 protein exclusively in hormone-producing cells, but not non-hormone producing folliculostellate cells in the anterior pituitary lobe. In addition, the cytoplasmic expression of UCH-L1 varied and was limited to gonadotropes and mammotropes. To investigate the role of UCH-L1 in the anterior pituitary cells, I performed a comparative analysis using wild-type and genetically UCH-L1-deficient *gad* mice. The numbers of gonadotropes and mammotropes in *gad* mice were obviously smaller than those in wild-type mice, although there was not difference in the number of other hormone-producing cells between wild-type and *gad* mice. The result suggests a close involvement of UCH-L1 in hormone production and/or development/maintenance of gonadotropes and mammotropes.

Introduction

The ubiquitin-proteasome system (UPS) is a major pathway for protein degradation to maintain normal cellular activities (Hochstrasser, 1995). A superfamily of proteins named deubiquitinating enzymes (DUBs) is involved in this process. Ubiquitin C-terminal hydrolases (UCHs) belong to DUBs, and at least four UCHs isozymes, which include UCH-L1, UCH-L3, UCH-L4 and UCH-L5 have been identified in mice. Among these isozymes, the expression and function of UCH-L4 and UCH-L5 are rarely known. On the other hand, mouse UCH-L1 and UCH-L3 share 52 % amino acid sequence identity (Kurihara et al., 2001; Osawa et al., 2001; Wilkinson, 1997). UCH-L3 is known to be expressed in almost all types of cells, whereas UCH-L1 was initially isolated from the brain, in which it was regarded as a neuronal marker and functioned as a monoubiquitin stabilizer (Doran et al., 1983; Osaka et al., 2003). In regard to its multifunction trait, UCH-L1 has been becoming one of the most dramatic proteins nowadays. There has been a close association of mutations in *Uchl1* gene with neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (Lowe et al., 1990; Setsuie and Wada, 2007). In addition, UCH-L1 was also reported to be expressed in a various types of tumor tissues (Campbell et al., 2003).

The anterior pituitary gland is an important component of the hypothalamic-pituitary-gonadal (HPG) axis. It consists of five distinct endocrine hormone-producing cell types, which include adrenocorticotropic hormone (ACTH) in corticotropes, growth hormone (GH) in somatotropes, prolactin (PRL) in lactotropes, thyroid-stimulating hormone (TSH) in thyrotropes and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in

gonadotropes, with a non-hormone producing cell type, the folliculostellate cells (FS cells). It has been reported that UCH-L1 is expressed in the anterior pituitary gland, suggesting particular functions of UCH-L1 in the organ, because UCH-L1 was selectively expressed unlike its isozyme UCH-L3 that was expressed ubiquitously (Kent and Rowe, 1992; Wilson et al., 1988). Among the components in the HPG-axis, it has been demonstrated the association of UCH-L1 with monoubiquitin in the neurons, in which UCH-L1 stabilized monoubiquitin, as well as the regulatory function of UCH-L1 in apoptosis in the testicular germ cells (Kwon et al., 2004b; Osaka et al., 2003). Furthermore, a novel role of UCH-L1 in polyspermy block has also been elucidated in mouse ova (Koyanagi et al., 2012; Sekiguchi et al., 2006). However, the precise distribution of UCH-L1 in the anterior pituitary gland has not yet been demonstrated in detail.

The gracile axonal dystrophy (*gad*) mouse is an autosomal recessive spontaneous mutant which has an intragenic deletion of the gene encoding mouse UCH-L1 (*Uchl1*). The deletion in *Uchl1* gene results in the systemic lack of the UCH-L1 protein expression (Saigoh et al., 1999). This mouse model has been broadly used to investigate the functional role of UCH-L1 in the nervous and reproductive systems. However, it remains unspecified what kinds of roles the UCH-L1 plays in the anterior pituitary gland in mice.

In this chapter, I attempted to determine the specific localization and expression pattern of UCH-L1 in mouse anterior pituitary gland. I found that UCH-L1 was expressed restrictedly in hormone-producing cells, but not non-hormone producing FS cells. Furthermore, the comparative analysis using wild type and UCH-L1-deficient *gad* mice indicated significant decreases in FSH cells, LH cells as well as PRL cells in *gad* mice,

suggesting the importance of UCH-L1 in these cells. These data might provide a new insight into the roles of UCH-L1 in the HPG-axis.

Materials and Methods

Animals

ICR male mice were purchased from Nihon SLC Inc. (Hamamatsu, Japan), and acclimated for 1 week. UCH-L1-deficient *gad* mice were obtained from National Institute of Neuroscience, National Center of Neurology and Psychiatry. The *gad* line was maintained by intercrossing for more than 20 generations as CBA and RFM mixed background. These mice were maintained at Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo. Animal care and handling were in accordance with institutional regulations and were approved by the Animal Care and Use Committee, The University of Tokyo.

Primary Antibodies

Rabbit polyclonal anti-UCH-L1 antibody was provided by Dr. Kwon (Chonbuk National University, Korea). Rabbit polyclonal anti-PGP 9.5 antibody was obtained from UltraClone (Wight, UK). Mouse monoclonal anti-PGP 9.5 antibody was obtained from Neuromics (Northfield, MN, USA). Mouse monoclonal anti-TSH, anti-ACTH, and rabbit polyclonal anti-GH, anti-S-100 antibodies were purchased from Dako (Glostrup, Denmark). Rabbit polyclonal anti-FSH, anti-LH and anti-PRL antibodies were purchased from Biogenisis (Poole, UK). Mouse monoclonal anti-β-actin antibody was from Sigma-Aldrich (St Louis, MO, USA).

Immunohistochemistry

Deparaffinized sections (2 μm thickness) were treated with absolute methanol containing 1% H_2O_2 for 30 min to block endogenous peroxidase activity. In order to enhance immunoreactivity, sections were subjected to autoclave treatment for 5 min at 100ºC. Non-specific binding was blocked by incubation with 100 % Block Ace (Dainippon Sumitomo Pharma Ltd., Osaka, Japan) for 1 h at room temperature. Then, the sections were incubated with primary antibodies against UCH-L1, FSH, LH, PRL and GH, respectively. The following day, sections were incubated with either biotinylated goat anti-rabbit or goat anti- mouse IgG antibody (DAKO Co., Glostrup, Denmark). After washing with PBS, the sections were incubated with streptavidin-biotin-horseradish peroxidase complex (sABC kit, DAKO Co., Glostrup, Denmark). Finally, the immunoreaction was visualized by incubation in 3, 3'-diaminobenzidine tetroxide (Sigma Chemical Co., St. Louis, MO, USA) and the sections were counterstained with hematoxylin.

Immunofluorescent staining

For immunofluorescent staining of pituitary tissue, experiments were performed in a standard method. Briefly, after antigen retrieval and blocking of non-specific binding, sections were incubated with anti-UCH-L1 and anti-hormone antibody or anti-S-100 antibody for 16 h at 4ºC. The following day, Alexa Fluor 488-labeled anti-rabbit IgG and Alexa Fluor 568-labeled anti-mouse IgG antibodies were incubated for 1 h at room temperature. Stained sections were mounted with mounting medium (DAKO, Glostrup, Denmark). Images were captured with a Zeiss LSM 510 confocal microscope.

Western blot analysis

Tissue extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gel for UCH-L1 protein. After being separated by electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 5% nonfat dry milk in PBS plus 1% Tween 20 (PBST) for 1 h at room temperature. The membranes were incubated with anti-UCH-L1 antibody (1:20,000), anti-β-actin antibody (1:20,000) as an internal control overnight at 4ºC. Then, the membranes were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000) or horseradish peroxidase-conjugated anti-mouse IgG (1:10,000) (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Immunoreactions were visualized by ECL plus (GE Healthcare, Piscataway, NJ, USA) and were detected using a CCD camera system (LAS-4000, Fujifilm, Tokyo, Japan).

Results

Expression of UCH-L1 in the anterior pituitary gland

To evaluate the expression level of UCH-L1 protein in the anterior pituitary gland, I performed a Western blot analysis with the anterior pituitary gland and other tissue extracts. The level of UCH-L1 in the anterior pituitary gland was extremely high, even significantly higher than that in the brain (Fig. 1-1). The UCH-L1 protein was not detected in protein extracts from the spleen, lung, liver as well as kidney. Furthermore, I conducted an immunohistochemical analysis to reveal the expression pattern of UCH-L1 in the pituitary gland (Fig. 1-2a). UCH-L1 immunoreactivity was detected in a large proportion of cells in the anterior lobe. In these cells, immunoreactive UCH-L1 was predominantly located in the nucleus with or without immunoreactive cytoplasm. On the other hand, some cells exhibited UCH-L1 immunoreactivity in the cytoplasm, but not in the nucleus (Fig. 1-2b, c). The cells in the intermediate lobe showed quite weak UCH-L1 immunoreactivity (Fig. 1-2d). In the posterior lobe, which is mainly composed of nerve terminals extended from the hypothalamus, UCH-L1 immunoreactivity was strongly expressed, but not in diffused pituicytes (Fig. 1-2e).

Localization of UCH-L1 protein in the anterior pituitary gland

The anterior lobe of pituitary gland consists of five different types of hormone-producing cells and non-hormone-producing FS cells. In an effort to investigate the cells in which UCH-L1 is expressed, I conducted a double-fluorescent staining to precisely position the localization of UCH-L1 protein in the anterior pituitary gland. As shown in Fig. 1-3,

UCH-L1 protein was co-stained with each hormone, respectively, as well as S-100, a marker for FS cells. Generally, UCH-L1 immunoreactivity was observed in the nuclei of six hormone-producing cells. However, the immunoreactivity of UCH-L1 in the cytoplasm showed relatively specific and distinctive pattern. UCH-L1 protein was expressed almost exclusively in the cytoplasms of many FSH-, LH- and PRL-producing cells (Fig. 1-3c, d, f), while not in those of TSH-, ACTH- and GH-producing cells (Fig. 1-3a, b, e). In addition, I did not observe UCH-L1 was co-expressed with FS cell marker S-100, which suggested UCH-L1 protein was not located in the non-hormone-producing cells (Fig. 1-3g).

Patterns of hormone-producing cells were altered in UCH-L1-deficient gad mice

I observed that UCH-L1 protein was exclusively expressed in hormone-producing cells in the anterior pituitary gland and the distribution of UCH-L1 was different among cell types. To assess function of UCH-L1, I compared hormone expression in the anterior pituitary cells between wild type (WT) and UCH-L1-deficient *gad* mice. As expected, the expression of UCH-L1 was not detected in homozygous *gad* mice (Fig. 1-4b). Immunohistochemical analyses were conducted with anti-FSH, LH, PRL and GH antibodies. A lot of GH-expressing cells were observed in the anterior pituitary glands and comparable in WT and *gad* mice (Fig. 1-4i, j). Although a modest number of FSH-, LH- and PRL-expressing cells were observed in WT mice (Fig. 1-4c, e, g), to my surprise, obviously decreased number of FSH-, LH- and PRL-expressing cells were observed in *gad* mice compared to those in WT mice (Fig. 1-4d, f, h).

Discussion

The ubiquitin-mediated protein degradation pathway is essential for eukaryotes and modulates many cellular processes (Hershko and Ciechanover, 1998). The proteins which are targeted for proteolysis are labeled with polyubiquitin chains and eventually degraded by the 26S proteasome (Thrower et al., 2000). After degradation of target proteins, DUBs regenerate polyubiquitin chains into individual ubiquitin molecules in order that they can be used again in the subsequent rounds. UCH-L1, a member of DUBs, is selectively and abundantly expressed in neurons and germ cells (Kon et al., 1999; Susor et al., 2007; Wilson et al., 1988). The HPG-axis is composed of three separate components which interact together to fulfill their assignments and are crucial to reproduction. Previous studies on UCH-L1 have mainly and intensively focused on its roles in neurons and genital organs of both sexes (Koyanagi et al., 2012; Kwon et al., 2004a; Sakurai et al., 2006; Yasuda et al., 2009; Yeung et al., 2006). However, the expression and the role of UCH-L1 in the pituitary gland have remained largely unknown. Although the anterior pituitary gland is an extremely small tissue in the body, it plays crucial roles in the endocrine system. Distinct hormone-producing cells cluster in the anterior lobe and regulate each of their downstream targets (Baker and Gross, 1978). In this chapter, I firstly confirmed the expression of UCH-L1 by Western blot analysis. UCH-L1 has been reported to be a key protein in the brain, not only its diverse functions, but also its abundance, accounting for approximate 1-2% of total proteins (Wilkinson et al., 1989; Wilson et al., 1988). Surprisingly, an extremely high expression level of UCH-L1 was detected in the anterior pituitary gland than that of brain extracts, which suggests the importance of UCH-L1 in the

anterior pituitary glands. By immunohistochemistry, I have shown that the majority of the anterior pituitary cells was immuno-positive for UCH-L1. However, it is hard to determine the types of cells expressing UCH-L1 by special location or cell shapes such as spermatocyte in the testis, or one-cell oocyte in the ovary. Here, I conducted immunofluorescent analyses to investigate the cell types in which UCH-L1 was expressed.

FS cells belong to a non-hormone producing cell type in the anterior pituitary gland (Horvath and Kovacs, 2002; Inoue et al., 1999). Except FS cells, UCH-L1 immunoreactivity was detected in the nuclei of all types of hormone-producing cells, and the expression of UCH-L1 in the cytoplasm was seen to be specific to FSH-, LH- and PRL-producing cells. These results suggest that UCH-L1 is involved in the hormones production or development and/or proliferation of FSH-, LH-, and PRL-producing cells.

gad mice are an autosomal recessive spontaneous mutant which is characterized with a "dying-back" type of axonal degeneration of the gracile tract. These mutant mice begin sensory ataxia at an early stage, followed by motor ataxia at a later stage. Around 6-month-old, mutant mice eventually die due to weakness. (Yamazaki et al., 1988b). Subsequent analysis revealed an intragenic deletion of *Uchl1* gene in this strain. Since *gad* mice do not express UCH-L1, they are considered as UCH-L1 null mutant mice (Saigoh et al., 1999). Previous studies have demonstrated that the lack of UCH-L1 resulted in an increase in abnormal spermatozoa, and a significantly increased rate of polyspermy in oocytes, respectively (Kwon et al., 2005; Sekiguchi et al., 2006). Furthermore, overexpression of UCH-L1 caused the inhibition of spermatogenesis, eventually leading to male infertility (Wang et al., 2006). These results suggest that the appropriate expression of UCH-L1 is

essential for reproduction. The anterior pituitary gland is an upstream tissue regulating terminal sexual organs. Alterations in the anterior pituitary gland would affect its regulation on the downstream tissues, which includes the testis and ovary. In this chapter, I have shown significant decreases in FSH- and LH-expressing cell numbers in *gad* mice, which might contribute to the defect in reproduction in *gad* mice (Yamazaki et al., 1988a).

I detected that the expression of UCH-L1 was in the nuclei of all six types of hormone-producing cells. However, cytoplasmic expression of UCH-L1 was only found in FSH-, LH- and PRL-producing cells. Subsequent analysis on *gad* mice revealed significant decrease in numbers of the cytoplasmic UCH-L1 expressing cells. I could not explain whether the specific expression of UCH-L1 was involved in the maintenance of these cells, and further study is needed to elucidate this issue. UCH-L1 is believed to hydrolyze the bonds between ubiquitin and small adducts *in vitro*, and the hydrolase activity of UCH-L1 is significantly lower than its isozyme UCH-L3 (Larsen et al., 1998). However, substrate(s) of this enzyme *in vivo* has not yet been identified. It is also necessary to be resolved whether some unknown substrates in the cytoplasm are linked with decreases in FSH-, LH- and PRL-producing cells in gad mice. In addition, a recently released report demonstrated that UCH-L1 functioned as a potentiator of cyclin-dependent kinases (CDKs) to enhance cell proliferation (Kabuta et al., 2013). However, the enhancement of UCH-L1 was dependent on interaction between UCH-L1 and CDKs, but not on its hydrolase activity. This also urges to figure out how UCH-L1 functions in the anterior pituitary cells.

In conclusion, I demonstrated the specific localization of UCH-L1 in mouse anterior pituitary gland for the first time and provided evidence that UCH-L1 might be involved in

hormone production or development and/or proliferation of FSH-, LH-, and PRL-producing cells.

Figures

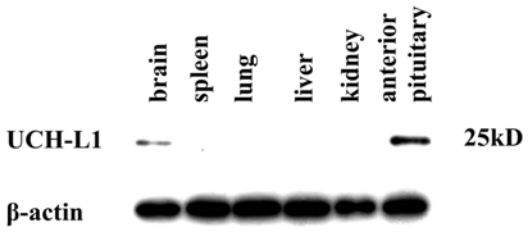

Figure 1-1. Western blot analysis of UCH-L1 protein expression in 8-week-old ICR mouse tissues. Various tissues as indicated from 8-week-old ICR mice were lysed and separated on 12.5% SDS-PAGE. β-actin was used as a control.

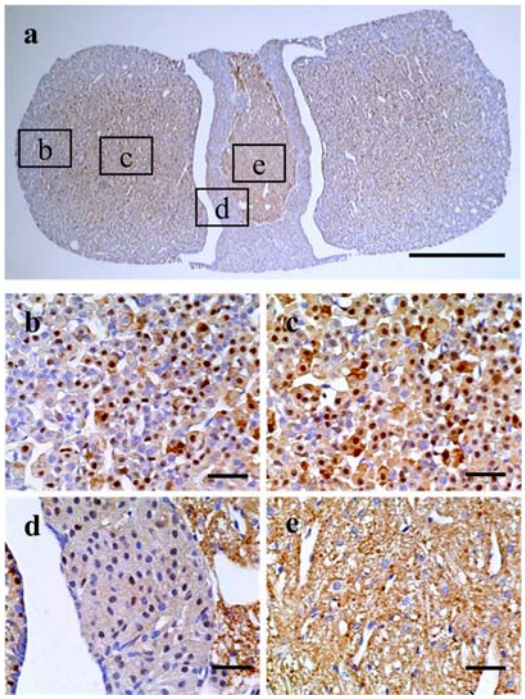

Figure 1-2. Immunohistochemical analysis of UCH-L1 protein distribution in 8-week-old ICR mouse pituitary gland. Pituitary glands from 8-week-old ICR mice were sectioned (2 μm thickness) to immunohistochemical analysis. (a) Overall immunoreactivity of UCH-L1 in the pituitary gland, bar=500μm. (b), (c), (d) and (e) High magnification of each rectangle as marked in (a), anterior lobe (b, c), intermediate lobe (d) and posterior lobe (e). bar=50μm.

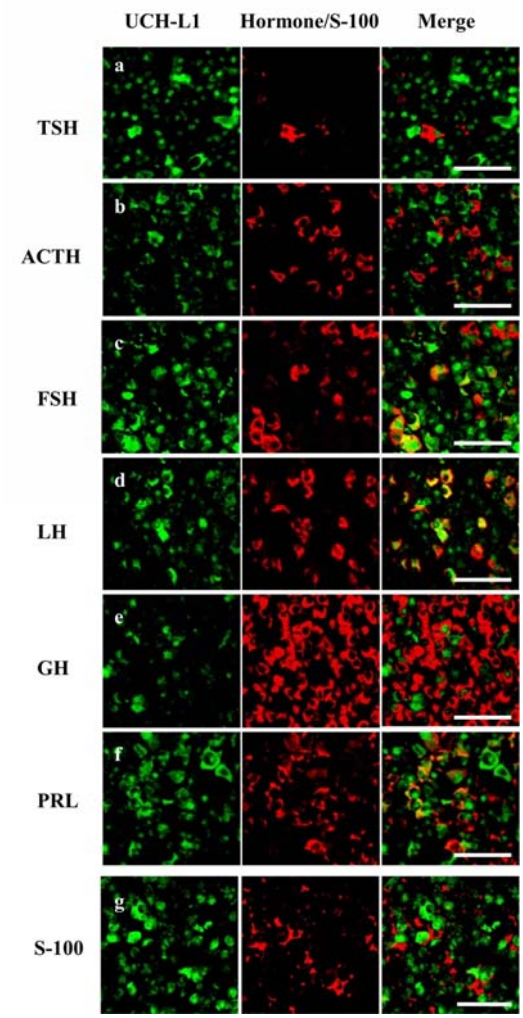

Figure 1-3. Immunofluorescent analysis of UCH-L1 localization in 8-week-old ICR mouse pituitary gland. Pituitary glands from 8-week-old ICR mice were sectioned (2 μm thickness) to immunofluorescent analysis. Double immunofluorescent staining of UCH-L1 protein (green) with each anterior pituitary hormone or FS cells marker S-100 (red). The immunofluorescence of UCH-L1 (left panels), pituitary hormones or S-100 (intermediate panels), and their merged images (right panels) are presented. TSH (a), ACTH (b), FSH (c), LH (d), GH (e), PRL (f) and S-100 (g). bar=50μm.

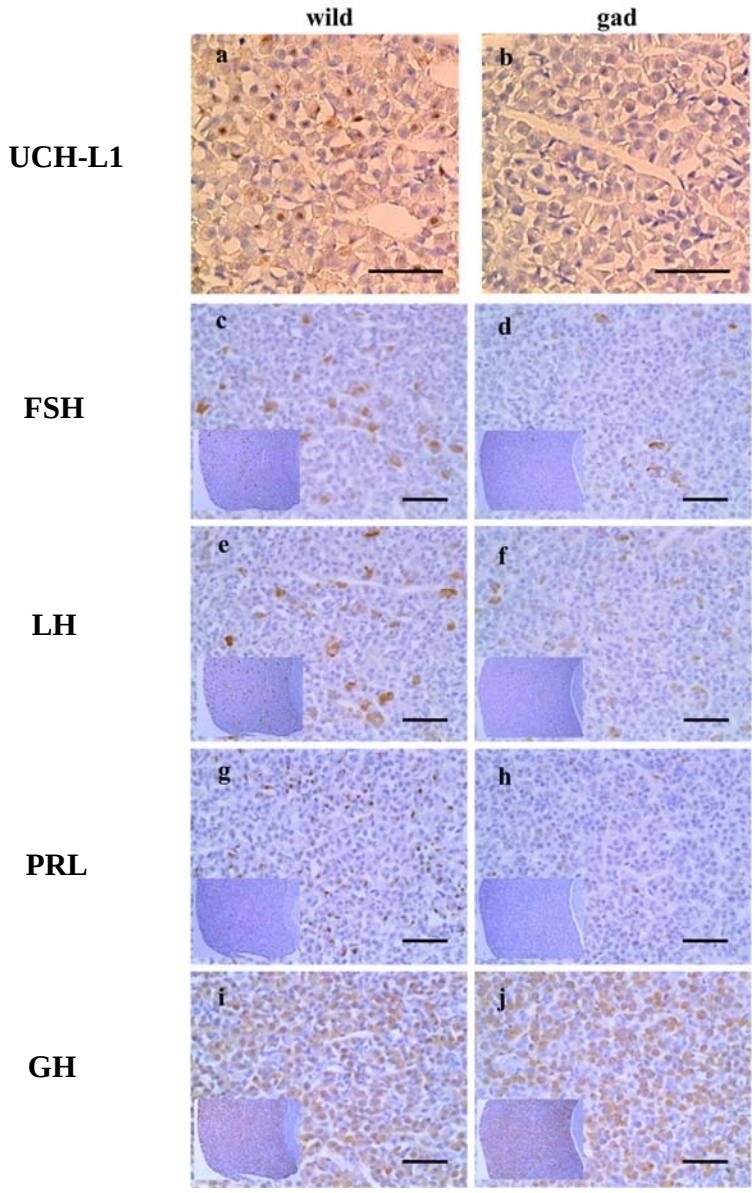

Figure 1-4. Immunohistochemical analysis of the anterior pituitary gland in wild type and UCH-L1-deficient *gad* mice. Pituitary glands from 8-week-old wild type (a) or *gad* mice (b) were sectioned (2 μm thickness) to immunohistochemical analysis of UCH-L1, bar=50μm. Immunohistochemistry of FSH (c, d), LH (e, f), PRL (g, h) and GH (i, j) in the

anterior pituitary glands of 22-week-old wild type (c, e, g and i) or *gad* mice (d, f, h and j), bar=50μm.

Chapter 2

The expression of Ubiquitin C-Terminal Hydrolase L1 in Gonadotrope Cell Lines and its involvement in apoptosis

Abstract

In chapter 1, significant decreases in the numbers of gonadotropes and mammotropes were observed in UCH-L1-deficient *gad* mice. UCH-L1 is an important protein in both testis and ovary. I had a special interest on the role of UCH-L1 in gonadotropes, because they are critical to reproduction. However, the anterior pituitary gland in mice is quite small and gonadotropes account for approximately 10% of anterior pituitary cell population. So, it is not easy to prepare a lot of murine gonadotropes to examine the role of UCH-L1. In this chapter, I chose gonadotropes cell lines αT3-1 cells and LβT-2 cells to examine the role of UCH-L1. Firstly, the morphologies of cultured cells and localizations of UCH-L1 were examined. I next examined the expression levels of UCH-L1 in both cell lines by RT-PCR and Western blotting. Apoptosis is a crucial cellular mechanism to maintain cell populations in tissues homeostatically. Several reports have demonstrated the involvement of UCH-L1 in apoptosis of neuronal and germ cells. I hypothesized the decrease in the number of gonadotropes in *gad* mice could be resulted from apoptosis. To examine whether UCH-L1 is involved in apoptosis of a gonadotrope cell line, LβT-2 cells were treated with a UCH-L1 specific inhibitor LDN-57444. The result showed that UCH-L1 inhibitor upregulated apoptosis of LβT-2 cells, suggesting that the deficiency of UCH-L1 might result in apoptosis of gonadotropes in *gad* mice.

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Chapter 3

Effect of Ubiquitin C-Terminal Hydrolase L1 on the expressions of gonadotropin genes by transient transfection assays

Abstract

To study the effects of a given gene, vector-mediated overexpression and siRNA-induced knockdown methods are frequently used, because protein expression level can be transiently up- or down-regulated. In this chapter, I used these technologies to examine whether the expression of gonadotropin genes would be affected by either upregulation or downregulation of UCH-L1 protein. In an attempt to do this, pcDNA 3.1 vector carrying full-length sequence of UCH-L1 coding region followed with a FLAG tag sequence or UCH-L1 specific siRNA was transfected into LβT-2 cells. As a result, the overexpression of UCH-L1 did not influence the transcriptions of all three subunits of gonadotropin genes, suggesting that sufficient UCH-L1 proteins had functioned in the cells. On the other hand, the inhibition of UCH-L1 protein expression by specific siRNA did not impact on mRNA expressions of each subunit of gonadotropin genes as well. These results suggest that the level of UCH-L1 does not affect transcriptional expressions of gonadotropin genes in LβT-2 cells. It might not be true that UCH-L1 is necessary in hormone production in gonadotropes in mice.

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General Conclusion

In these studies, I have firstly demonstrated the specific localization and expression pattern of UCH-L1 in mouse anterior pituitary gland *in vivo* and gonadotrope cell lines *in vitro*. Furthermore, I have demonstrated the function of UCH-L1 using UCH-L1-deficient *gad* mice and gonadotrope cell lines.

UCH-L1 has been emerging as an important protein in both testis and ovary, which suggests its close involvement related to the reproductive system. Anterior pituitary gland is a major component for reproduction, secreting hormones that regulate downstream sexual tissues. UCH-L1 was initially identified as PGP 9.5. Although it has been detected by immunohistochemical analysis in the anterior pituitary gland in 1988, that report did not provide sufficient information on its expression as well as function. Therefore, it is interesting and necessary to examine the specificity of UCH-L1 in the anterior pituitary gland.

In Chapter 1, I demonstrated that UCH-L1 was abundantly expressed in mouse anterior pituitary gland. The localization of UCH-L1 was restricted to hormone-producing cells, but not non-hormone producing FS cells. The expression of UCH-L1 was in the nuclei of all six types of hormone-producing cells, while the cytoplasmic expression of UCH-L1 was only found in FSH-, LH- and PRL-producing cells. To elucidate the function of UCH-L1 in mouse anterior pituitary gland, I performed a comparative analysis using wild type and UCH-L1-deficient *gad* mice. Significant decreases in the numbers of FSH cells, LH cells and PRL cells were observed in *gad* mice. These results suggest the importance of UCH-L1 in these cells.

FSH and LH are synthesized and secreted from the same cells, gonadotropes. I focused

on the functional role of UCH-L1 in gonadotropes since these cells have a close involvement in the reproduction. Based on the observations in *gad* mice, I hypothesized that decrease in the number of gonadotropes might be resulted from apoptosis or failure in the expressions of gonadotropin genes. However, mouse anterior pituitary gland is a quite small tissue and gonadotropes constitute only 10 % of anterior pituitary cell population. To test my hypotheses, as an alternative approach, I chose gonadotrope cell lines to examine the role of UCH-L1 *in vitro*.

In Chapter 2, I demonstrated that UCH-L1 was also expressed in gonadotrope cell lines, αT3-1 and LβT-2 cells. The localization of UCH-L1 was found in both nuclei and cytoplasm in these cell lines, which was consistent with *in vivo* results in Chapter 1. αT3-1 and LβT-2 cells represent immature and mature types of gonadotropes, respectively. I examined the expressions of UCH-L1 and its isozyme, UCH-L3. Although the protein expression levels of these two isozymes did not have a significant difference, distinct mRNA expressions of UCH-L1 and UCH-L3 were detected in these cell lines. These results suggested their different requirements during development of gonadotropes. Furthermore, I treated LβT-2 cells with a UCH-L1 specific inhibitor, LDN-57444. Apoptosis was largely induced in LDN-57444-treated cells. These results suggest that the deficiency of UCH-L1 resulted in apoptosis of gonadotropes in *gad* mice.

In Chapter 3, I examined whether UCH-L1 had an effect on the expressions of gonadotropin genes using LβT-2 cells. I performed transient transfections to upregulate and to downregulate UCH-L1 protein. Overexpression of UCH-L1 did not influence the transcriptions of all three subunits of gonadotropin genes. On the other hand, siRNA-

induced knockdown of UCH-L1 protein also did not alter the transcriptions of gonadotropin genes. However, the efficiency of knockdown of UCH-L1 was not sufficient, further study might be required to solve this problem. In the present study, my results suggest that the level of UCH-L1 did not affect the transcriptional expressions of gonadotropin genes in LβT-2 cells. And it also suggest that UCH-L1 might not be necessary in hormone production in gonadotropes in mice, although it might be necessary in the survival.

Anterior pituitary gland builds a linkage between the nervous and reproductive systems. The function of UCH-L1 has been well demonstrated in these two systems, however, the role of UCH-L1 in the anterior pituitary gland is still largely unknown. Anterior pituitary gland is composed of distinct types of hormone-producing cells. This thesis provided a fundamental basis to study the function of UCH-L1 in these hormone-producing cells. I demonstrated that the function of UCH-L1 in mouse anterior pituitary gland and gonadotrope cell lines. These results indicated that UCH-L1 is a crucial protein in gonadotropes, providing a novel insight for better understanding the role of UCH-L1 in the hypothalamic-pituitary-gonadal axis and in the reproduction. Additional studies are necessary to determine the function of UCH-L1 in other types of hormone-producing cells in the anterior pituitary gland.

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