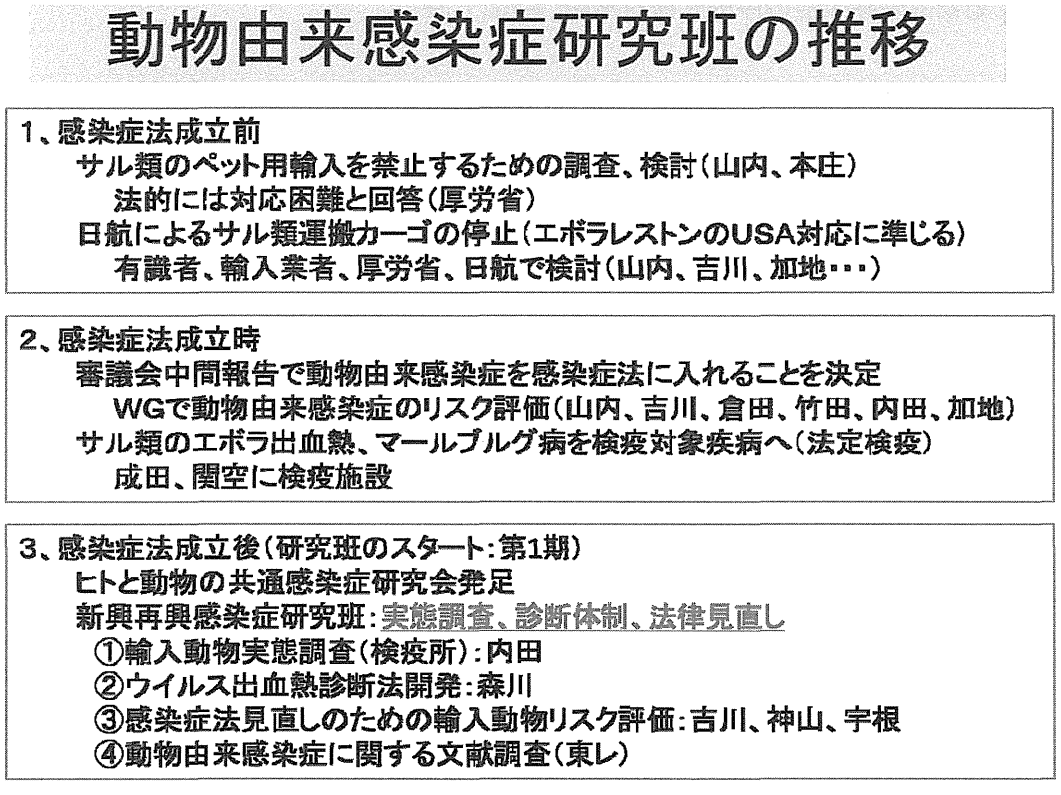
2. 動物由来感染症のサーベイランスに関する論点整理

2.1 班会議における動物由来感染症研究に関する議論

2014 年 8 月に開催された班会議、9 月に開催された統括班打合せ、12 月に開催された班会議においては、動物由来感染症研究について、吉川班長からのこれまでの取り組みの経緯の振り返りが行われ、班員によるディスカッションが行われた。



4、新興再興感染症研究班第2期：財務省データ、獣医師・医師調査、1類疾患診断法
①輸入動物の財務省（関税）による新分類のためのデータ作成（太田、吉川）
②獣医師、動物看護士へのアンケート調査、医師への動物由来感染症調査（内田）
③輸入動物由来感染症WGリスク評価（宇根、神山、吉川）
④エボラ、マールブルグ、ラッサ熱等の診断法確立（森川）
輸入動物の禁止、検疫、届出制を法制化

5、新興再興感染症研究班第3期：病原体保有調査、港湾労働者リスク、序列化開始
①港湾労働者への感染症アンケート調査（内田）
②輸入動物のトレーサビリティー制度（太田）
③輸入動物の疾病、病原体保有調査（宇根、丸山）
④動物由来感染症の序列化検討開始（吉川、門平）

6、新興再興感染症研究班第4期：5研究班統合、感染症総合危機管理、序列化
①動物由来感染症の序列化（AHP法：門平、吉崎、太田、吉川）
②輸入動物、野生動物、展示動物（ウイルス、細菌、寄生虫）の統合
（神谷、杉山、井上、今岡、宇根、丸山・・・・）
③海外調査（中国、フィリピン、欧米諸国ほか杉山、吉川、宇根、門平、：）
④狂犬病ガイドライン改訂（井上）

4、新興再興感染症研究班第2期：財務省データ、獣医師・医師調査、1類疾患診断法
①輸入動物の財務省（関税）による新分類のためのデータ作成（太田、吉川）
②獣医師、動物看護士へのアンケート調査、医師への動物由来感染症調査（内田）
③輸入動物由来感染症WGリスク評価（宇根、神山、吉川）
④エボラ、マールブルグ、ラッサ熱等の診断法確立（森川）
輸入動物の禁止、検疫、届出制を法制化

5、新興再興感染症研究班第3期：病原体保有調査、港湾労働者リスク、序列化開始
①港湾労働者への感染症アンケート調査（内田）
②輸入動物のトレーサビリティー制度（太田）
③輸入動物の疾病、病原体保有調査（宇根、丸山）
④動物由来感染症の序列化検討開始（吉川、門平）

6、新興再興感染症研究班第4期：5研究班統合、感染症総合危機管理、序列化
①動物由来感染症の序列化（AHP法：門平、吉崎、太田、吉川）
②輸入動物、野生動物、展示動物（ウイルス、細菌、寄生虫）の統合
（神谷、杉山、井上、今岡、宇根、丸山・・・・）
③海外調査（中国、フィリピン、欧米諸国ほか杉山、吉川、宇根、門平、：）
④狂犬病ガイドライン改訂（井上）

ディスカッションにおいては、

○動物由来感染症については、動物側の調査は難しく、これまではパッシブサーベイランスが主体であり、散発的な対策にとどまってきた、
○動物由来感染症がヒトに来るときについてのリスク評価は、本研究班において行ってきたが、実際に動物由来感染症の病原体が、リザーブされている野生動物において、どのような振る舞いをしているか、についてモニタリングを行い、理解を深めていくことが予防には重要である、
○昨今の国際情勢(アグリテロ、バイオテロ)を踏まえ、通常の病原体と鑑別する研究開発も必要であろう、
○AHP で重要と評価されるもの、国際的なテロ対策 などの議論をふまえ、モデルケースとしていくつかの病原体を取り上げての取り組みが必要ではないか。
○アクティブサーベイライスを実施するためには、その効率的な仕組み作りも模索していく必要があるのではないか、平常時のモニタリングとして継続していける仕組み作りが不可欠ではないか。
○GIS を含めたデータの集積・蓄積、必要時の積極的介入により、システムの有効性を示していくことができればよい。

との意見が得られた。

2.2 動物由来感染症サーベイランスの例

研究班におけるディスカッションをふまえ、動物由来感染症をターゲットとしてたサーベイランスの取り組みについて、調査を行った。

ヒトや家畜の感染症に関する個別のサーベイランスは、各国において積極的に実施、データの公表もなされているが、ヒトと動物の両方のデータを統合したシステムはまだ少ない。Wendt ら[1]は、文献調査により、以下の基準に基づき 20 のサーベイランスシステムを抽出し、まとめている。

1．予防または人獣共通感染症を制御することを意図して開発されたもの
2．ヒトおよび動物からの情報が含まれている限りにおいて、ヒトおよび動物の健康コミュニティ間の分野横断的な協力を遂行するもの
3．情報システムにおける健康情報を共有するもの
4．異なる特性（異種データ）を有するさまざまなソースからのモニタリングと監視データを統合したもの

ヒトおよび動物に関する情報を集積する疫学的研究のための一時的なデータ収集、一般には臨床試験とバイオバンクなどは含めない。

表1には、ヒトと動物の健康情報を統合した14のサーベイランスシステム、ヒトと動物の健康情報を統合した6つの症候群サーベイランスについて、一部情報を追加して日本語でまとめたものを示した。

Wendt ら[1]によると、これら 20 のうち 14 のシステムが 2006 年以降に開発され、6 のシステム(ABIN、CAHSN、Global Food Safety Portal、INDICATOR、NBIC、NCB-Prepared)は、現時点でも確立途中とのことである。

それぞれの URL や紹介ページを確認したが、クローズドな DB である場合も多く、個別の情報例までは確認できなかった。しかし、これまで同じプラットフォームに上げる試みが少なかったヒトと動物（あるいは食品も含む）の疾病関連データを統合して集積・分析する試みが各国で進められていることは画期的であり、情報化社会の発達を背景に、動物由来感染症の伝播や早期対応についての研究がより進んでいくと期待される。

1 Wendt, A., Kreienbrock, L. and Campe, A. (2015), Zoonotic Disease Surveillance - Inventory of Systems Integrating Human and Animal Disease Information. Zoonoses and Public Health, 62: 61-74. doi: 10.1111/zph.12120

表 1 ヒトと動物の健康情報を統合した 14 のサーベイランスシステム、ヒトと動物の健康情報を統合した 6 つの症候群サーベイランス（参考文献 1 を基に作成、追記）

システム名	URL	参加機関	システムの種類	開始年	サーベイランス地域	疾病数	疾病の発生	疾病/病原体	モニター対象	サーベイランスの目的	興味のあるデータ	データ元/データ提供者	二次データ
ABIN(The Australian Biosecurity Intelligence Network)	https://www.abin.org.au/	オーストラリア政府国家共同研究	人間と動物の健康をカバーする、統合化されたバイオセキュリティ基盤を確立するためのネットワーク(人間と動物の健康をカバーする)	2008	オーストラリア	広範囲	限定されない	適用せず	ヒト; 動物; 植物	早期検出;モニタリング; 理解向上	不明	不明	有り
AFHSC-GEIS(Armed Forces Health Surveillance Center (Global Emerging Infections Surveillance and Response System))	http://www.afhsc.mil/home	AFHSC; DOD	同時に行われるハイリスク群におけるサーベイランスとともに新興感染症における備えを促進するためのプログラム	2008	アジア;アフリカ;東ヨーロッパ;旧ソビエト連邦; 南アメリカ西部	広範囲	新興	適用せず	ヒト; 動物; ベクター	予測; 早期検出	ラボ結果; 診断	研究室;監視サイトからの作業者	なし
ArboNET	URL 不明	CDC; U.S. Geological Survey (USGS)	アルボウイルス病のために国レベルのサーベイランス取り組みを統合するための CDC データベース	2000	米国	集中的	地域流行	アルボウイルス感染症	ヒト; 動物; ベクター	予測; 早期検出; モニタリング	疾病発生についての報告あるいは通知; ラボ結果	州および地方健康局	有り
ArboZoonet	(Final Meeting が 2012 開催、現在リンク切れ)	OIE; FAO; ProMED	新興ウイルス性節足動物媒介性疾病のコントロールの能力形成のためのネットワーク	2008	欧州;中国; トルコ;イラン; 南アフリカ	集中的	新興	アルボウイルス感染症	ヒト; 動物; ベクター	モニタリング; 理解向上	疾病発生についての報告あるいは通知	公式の通知; 公表論文;その他入手可能な論文	有り
CAHSN(Canadian Animal Health Surveillance Network)	URL 不明	NCFAD Winnipeg; CNPHI	リアルタイムで新興の動物疾病の脅威を検出するための動物の健康診断ラボのネットワーク	2005	カナダ	広範囲	新興	適用せず	農場動物; ヒト	早期検出;モニタリング	ラボ結果; 疑い症例の報告	動物の健康にかかわる研究室; アカデミックの究室; CFIA 研究室; 開業獣医	有り

システム名	URL	参加機関	システムの種類	開始年	サーベイランス地域	疾病数	疾病の発生	疾病/病原体	モニター対象	サーベイランスの目的	興味のあるデータ	データ元/データ提供者	二次データ
CDAP(Canada Database of Animal Parasites)	URL 不明	CFIA	特定の寄生虫についての全国データを整理、分析、提供するためのデータベース	1999	カナダ	集中的	地域流行	7つの人獣共通感染寄生虫	ヒト; 動物; 食品	モニタリング; 理解向上	疾病発生についての報告あるいは通知	出版物;政府の記録; 背景データ	有り
Disease Bioportal	http://bioportal.ucdavis.edu/about	カリフォルニア大学デービス校; WHO; FAO; OIE	リアルタイムもしくはほぼリアルタイムで地域、地方、グローバルの疾病情報を提供するウェブベースのシステム	2007	全世界	広範囲	限定されない	適用せず	ヒト; 動物	早期検出;モニタリング	ラボ結果; 疾病発生についての報告あるいは通知	Empres-I; OIE and WHO RVF reports; PANAFTOSA; FMD Gene Bank; UC Davis FMD news	有り
EIDSS(The Electronic Integrated Disease Surveillance System)	https://eidss.codeplex.com/	Ministries of Health and Agriculture; CDC	全国レベルで　疾病のアウトブレイク疑いについて迅速な報告を提供するためのデータマネージメントシステム	2007	中央アジア; コーカサス	広範囲	限定されない	適用せず	ヒト; 動物	モニタリング	疾病発生についての報告あるいは通知; 疑い症例の報告; ラボ結果	病院; 健康関連施設;獣医診療; 研究室; 農場; 監視サイト	なし
GLEWS(The Global Early Warning System)	http://www.glews.net/	WHO; FAO; OIE	OIE と FAO、WHO のアラートと疾病の情報体系を結びつけるシステム	2006	全世界	広範囲	限定されない	適用せず	ヒト; 動物	予測; 早期検出理解向上	疾病発生についての報告あるいは通知; 疑い症例の報告	WAHID; Empres-I; WHO Global health Atlas; UN data; ProMED; GPHIN	有り

システム名	URL	参加機関	システムの種類	開始年	サーベイランス地域	疾病数	疾病の発生	疾病/病原体	モニター対象	サーベイランスの目的	興味のあるデータ	データ元/データ提供者	二次データ
Global Food Safety Portal	不明	National Food Institute Denmark; WHO	他のモニタリングプログラムより食品とコンテキストデータについての情報を統合し、提示するウェブベースのポータルサイト	2010	全世界	集中的	地域流行	食品媒介疾病	ヒト; 動物; 食品	理解向上	疾病発生についての報告あるいは通知	モニタリングプログラムからの入手可能な公開データ; WHO Global Food Network Country database	有り
INDICATOR	不明	Health Information Technology Centre University Illinois	サイバーインフラによる、すべての情報源からのルーチンサーベイランスデータ	2008	イリノイ州(米国)	広範囲	限定されない	適用せず	ヒト; 動物; 環境	早期検出;モニタリング	疾病発生についての報告あるいは通知; ラボ結果; indirect indicators	病院; 法定疾病のデータベース;動物診療所; 研究室;天気レポート	有り
ISS	URL 不明	BCCDC; PHAC; BCMAg; CFIA	フードチェーンに添った食中毒菌の統合されたサーベイランスシステム	2006	ブリティッシュコロンビア州(カナダ)	集中的	地域流行	サルモネラ	ヒト; 動物; 食品	Identifying health risks;モニタリング	ラボ結果	動物健康センター; CFIA; BCCDC; PHMRL; PHAC	有り
NBIC(National Biosurveillance Integration Center)	URL 不明	DHS	データの統合と専門化による分析によってバイオサーベイランスの有効性を高めるための複数機関によるコラボレーション	2007	全世界	広範囲	限定されない	適用せず	ヒト; 動物; 食品; 環境	早期検出	疾病発生についての報告あるいは通知; 疑い症例の報告	公的機関;世界ニュースメディア;公表されているウェブサイト	有り
NCB-Prepared (The National Collaborative for Bio-Preparedness)	http://www.ncb-prepared.org/	北カリフォルニア福祉局	ヒトから動物、食品由来のデータを分析するための州全体のバイオサーベイランスシステム	2008	北カリフォルニア(米国)	広範囲	限定されない	適用せず	ヒト; 動物; 食品; 環境	早期検出	疾病発生についての報告; indirect indicators	病院; 医師; 獣医師;食糧期間	有り

システム名	URL	参加機関	システムの種類	開始年	サーベイランス地域	疾病数	疾病の発生	疾病/病原体	モニター対象	サーベイランスの目的	興味のあるデータ	データ元/データ提供者	二次データ
BioCaster	http://biocaster.nii.ac.jp/index.php?lang=jp(リンクなし)	国立情報学研究所(日本)	新規のアウトブレイクについて情報を収集、提供するためのインターネットベースの自動システム	2006	アジア太平洋地域	広範囲	限定されない	適用せず	ヒト;動物;植物;環境	早期検出	疑い症例の報告	ニュース記事(EurekAlert!, EMM Alerts, Google, CDC's weekly reports, Meltwater, OIE, ProMED, Reuters, WHO, VetSweb)	有り
EpiSPIDER	www.epispider.org/	米国政府	統合された視点による報告衛生事例の考察を提供するための異種の電子情報源を統合するオープンアクセスのアプリケーション	2006	全世界	広範囲	限定されない	適用せず	ヒト;動物	早期検出	疑い症例の報告	ニュース記事(Daylife, Google, HumanitarianNews, Moreover, ProMED, Twitter and WHO)	有り
GPHIN(The Global Public Health Intelligence Network)	(要認証)https://www.gphin3.net/About/Details/2	カナダ政府;WHO	新規のメディア情報源より公衆衛生関連の予備的なレポートを収集するためのインターネットベースのシステム	1997	全世界	広範囲	限定されない	適用せず	ヒト;動物;食品;環境	早期検出	疑い症例の報告	ニュースフィード集(Factiva, Al Bawaba)	有り

3. 研究班総合会議、研究統括班会議等の運営及び報告書等の資料作成支援

3.1 会議等の支援

研究班総合会議の開催(研究コーディネーター、厚労省行政官、班員)の運営、研究統括班の開催及び研究統括班の開催及び研究分担班の会議の運営を支援し、必要に応じてその他の会議等の資料作成を支援した。

表 3-1 会議等の運営・資料作成支援

会議等(場所)	開催月	資料作成等
統括班会議 (東京都内)	2014 年 6 月	開催支援、資料作成支援等*
B ウイルス会議 (東京都内)	2014 年 8 月	開催支援*
班会議 (東京都内)	2014 年 8 月	開催支援、資料作成支援等*
統括班打合せ (東京都内)	2014 年 9 月	開催支援*
班会議 (東京都内)	2014 年 12 月	開催支援、資料作成支援等
班長打ち合わせ (東京都内)	2014 年 1 月	打ち合わせ

(*オブザーバーとして参加)

3.2 研究統括班への協力

研究統括班に協力し、各研究を遂行させるためのコーディネート、支援を行った。

以上

Ⅳ.　業績資料集

Exp. Anim. 63(2), 247–256, 2014

—Original—

Ubiquitin C-Terminal Hydrolase L1 Is Expressed in Mouse Pituitary Gonadotropes *In Vivo* and Gonadotrope Cell Lines *In Vitro*

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Abstract: The ubiquitin-proteasome system (UPS) plays a fundamental role in regulating various biological activities. Ubiquitin C-terminal hydrolase L1 (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 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 the present study, we 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 anterior pituitary cells, we performed a comparative analysis using genetically UCH-L1-deficient *gad* mice. Significant decreases in the numbers of gonadotropes and mammotropes were observed in *gad* mice, suggesting a close involvement of UCH-L1 in these cells. Moreover, we also determined the expression of UCH-L1 in cultured gonadotropes. Taken together, this is the first report to definitely demonstrate the presence of UCH-L1 in mouse anterior pituitary gland, and our results might provide a novel insight for better understanding the role of UCH-L1 in the hypothalamic-pituitary-gonadal axis and in the reproduction.

Keywords: αT3-1 cells, *gad* mice, LβT-2 cells, pituitary gland, UCH-L1

Introduction

The ubiquitin-proteasome system (UPS) is a major pathway for protein degradation to maintain normal cellular activities [7]. 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 [15, 23, 33]. 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 [4, 22]. 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 [21, 28]. In addition, UCH-L1 was also reported to be expressed in a various types of tumor tissues [3].

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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 [12, 34]. 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 [17, 22]. Furthermore, a novel role of UCH-L1 in polyspermy block has also been elucidated in mouse ova [14, 27]. 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 [25]. 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 the present study, we attempted to determine the specific localization and expression pattern of UCH-L1 in mouse anterior pituitary gland. We 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.

Cell cultures and preparation

αT3-1 and LβT-2 cells were generous gifts from Prof. Pamela Mellon (University of California, San Diego, CA, USA) [1, 24]. Both cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical, Ltd., Tokyo, Japan), supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 IU/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in 95% air. Cells were seeded and cultured in 6-well culture plates (Thermo Scientific, Rochester, NY, USA) or 8-well culture slides of Lab Tek II Chamber (Thermo Scientific) for experiments.

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). Rabbit polyclonal anti-UCH-L3 antibody was obtained from Cell Signaling (Beverly, MA, 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).

Table 1. Primer sets for semi-quantitative RT-PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Length[1)]
Mouse *Uchl1*	CCGTCCTGAAACAGTTTCTG	AGCTGCTTTGCAGAGAGCCA	335
Mouse *Uchl3*	GGAGCCTGAACTTCTTAGCATGG	TGGATTAGTCCAATCGTTCCACA	195
Mouse *Uchl4*	GCCTGTGGAACGATTGGAACGATT	CCACCACTGCTGGGCTATTCTTGT	622
Mouse *Uchl5*	GGTCCAGGACTCCAGACTTGAA	CCCTCTCTTAACCCGTCTAGTT	348
Mouse common-α-subunit	GCAGCTGTCATTCTGGTCATG	CGACTTGTGGTAGTAGCAAG	339
Mouse *Fshb*	AGCACTGACTGCACCGTGAG	CCTCAGCCAGCTTCATCAGC	606
Mouse *Lhb*	GCCTGTCAACGCAACTCTGG	CAGGCCATTGGTTGAGTCCT	300
Mouse *Gapdh*	GTCTTCACCACCATGGAGAA	ACAACCTGGTCCTCAGTGTA	545

[1)] Length of product after PCR amplification.

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 100°C. 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.). After washing with PBS, the sections were incubated with streptavidin-biotin-horseradish peroxidase complex (sABC kit, DAKO Co.). Finally, the immunoreaction was visualized by incubation in 3, 3'-diaminobenzidine tetroxide (Sigma Chemical Co.) 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 4°C. 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). Images were captured with a Zeiss LSM 510 confocal microscope.

For immunofluorescent staining of cultured cells, αT3-1 and LβT-2 cells were seeded in 8-well culture slides 24 h prior to experiment at a density of 1×10^4 cells/well. Then, these cells were fixed in 4% paraformaldehyde/PBS for 15 min. After washing cells with PBS three times, cells were permeabilized with 0.1% Triton X-100/PBS for 20 min. Nonspecific binding was blocked by incubating with Block Ace for 1 h at room temperature. Then, they were treated with primary antibody against UCH-L1 diluted at 1:1,000 at 4°C overnight. After washing three times with PBS, Alexa Fluor 488-labeled goat anti-rabbit IgG antibody diluted at 1:1,000 in PBS was added to these samples for 1 h at room temperature. To visualize the nuclei, To-Pro-3 iodide (Life Technologies, Carlsbad, CA, USA) in PBS at a dilution of 1:1,000 was introduced into these samples together with secondary antibody. After washing with PBS, these samples were immersed with VectaShield (Vector Laboratories Inc., Burlingame, CA, USA) and covered with coverslips. Finally, the stained cells were photographed under a Zeiss LSM 510 confocal microscope.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from αT3-1 and LβT-2 cells using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. For semi-quantitative RT-PCR, the total RNA was reverse transcribed using Superscript III reverse transcriptase (Life Technologies) with oligo (dT) primers (Life Technologies) according to the manufacturer's instruction. Then, the expressions of mRNA were monitored by RT-PCR. The primer sets used in this experiment are listed in Table 1. RT-PCR was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by gene specific cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, followed with a final extension at 72°C for 5 min. Data was normalized to expression level of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Western blot analysis

Tissue extracts or cell lysates were subjected to so-

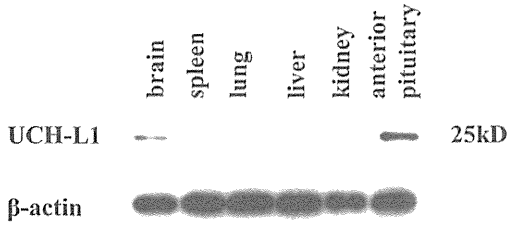

Fig. 1. Western blot analysis of UCH-L1 protein expression in 8-week-old ICR mouse tissues. Various tissues as indicated from 8-week-old ICR mice were lysed and separated on 12.5% SDS-PAGE. β-actin was used as a control.

dium 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-UCH-L3 antibody (1:1,000) or anti-β-actin antibody (1:20,000) as an internal control overnight at 4°C. 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) 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).

Statistical analysis

Normalized data on mRNA and protein expressions are shown as the means with standard error of the means (SEM). Statistical analysis was performed with Student's *t*-test for comparisons between groups using Microsoft Excel software. Values of $P<0.05$ were considered statistically significant.

Results

Expression of UCH-L1 in the anterior pituitary gland

To evaluate the expression level of UCH-L1 protein in the anterior pituitary gland, we 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). The UCH-L1 protein was not detected in protein extracts from the spleen, lung, liver as well as kidney. Furthermore, we conducted an immunohistochemical analysis to reveal the expression pattern of UCH-L1 in the pituitary gland (Fig. 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. 2b and c). The cells in the intermediate lobe showed quite weak UCH-L1 immunoreactivity (Fig. 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. 2e).

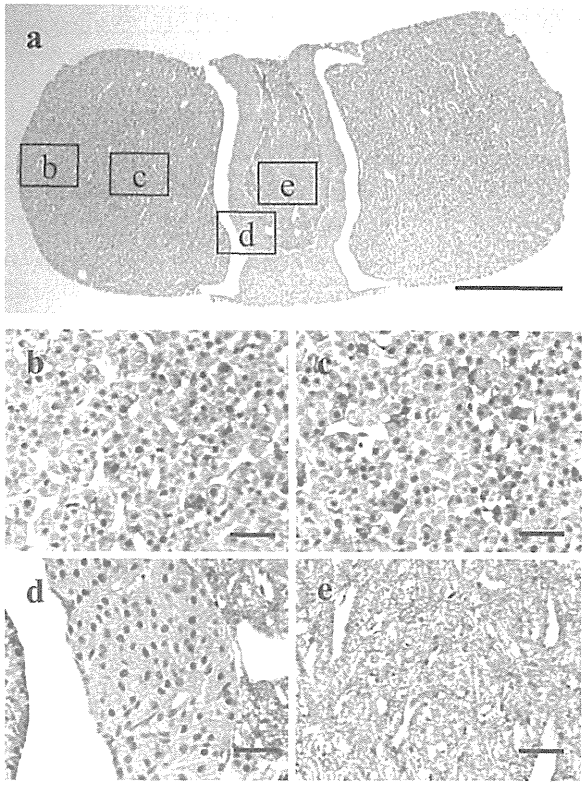

Fig. 2. Immunohistochemical analysis of UCH-L1 protein distribution in 8-week-old ICR mouse pituitary gland. Pituitary glands from 8-week-old ICR mice were sectioned (2 μm thickness) to immunohistochemical analysis. (a) Overall immunoreactivity of UCH-L1 in the pituitary gland, bar=500 μm. (b), (c),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

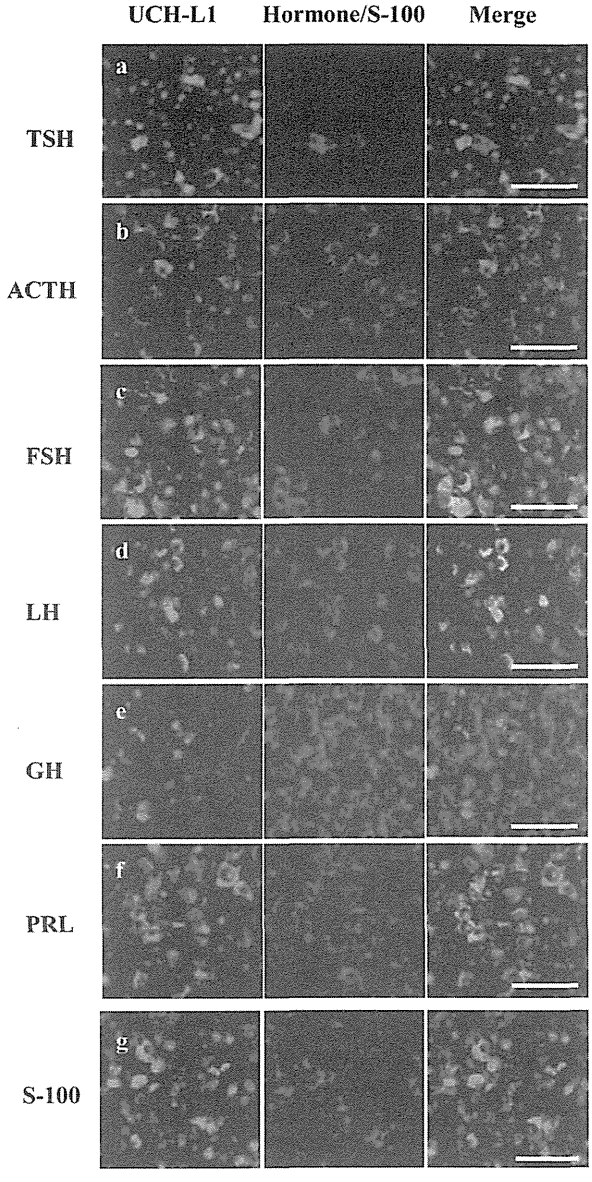

Fig. 3. Immunofluorescent analysis of UCH-L1 localization in 8-week-old ICR mouse pituitary gland. Pituitary glands from 8-week-old ICR mice were sectioned (2 μm thickness) to immunofluorescent analysis. Double immunofluorescent staining of UCH-L1 protein (green) with each anterior pituitary hormone 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=50 μm.

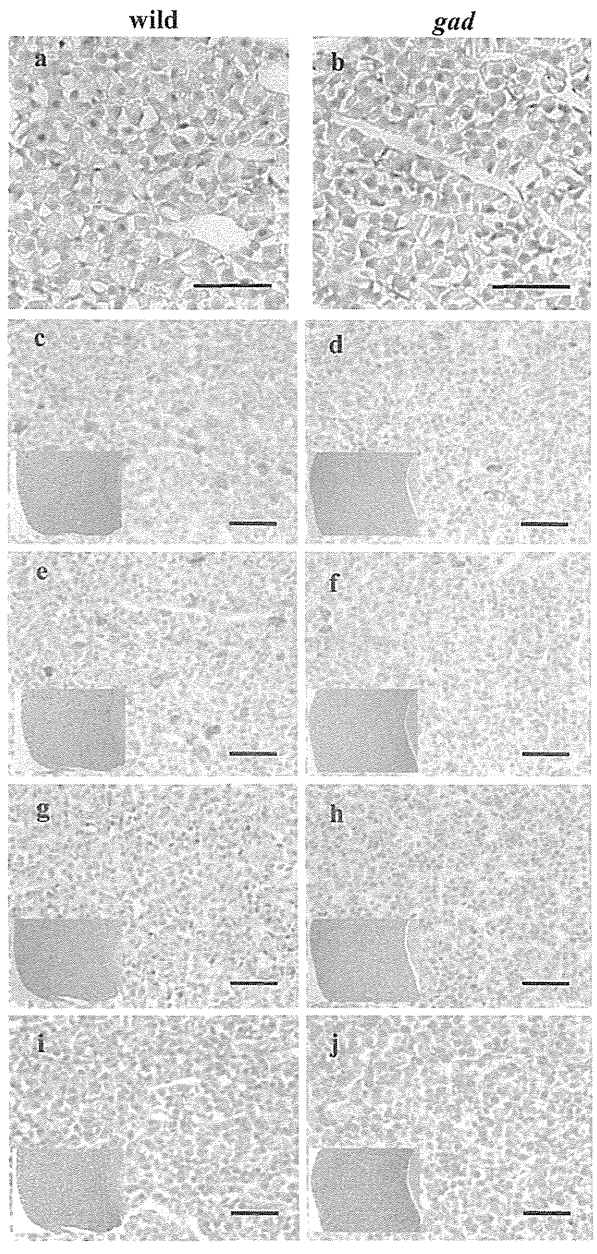

Fig. 4. Immunohistochemical analysis of the anterior pituitary gland in wild type and UCH-L1-deficient *gad* mice. Pituitary glands from 8-week-old wild type (a) or *gad* mice (b) were sectioned (2 μm thickness) to immunohistochemical analysis of UCH-L1, bar=50 μm. Immunohistochemistry of FSH (c, d), 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=50 μm.

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, we conducted

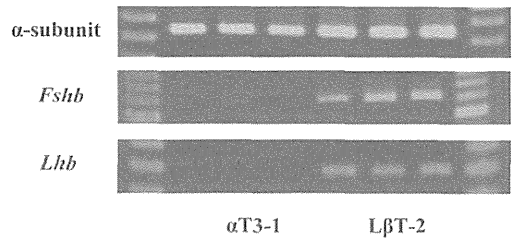

Fig. 5. Confirmation on expressions of three subunits of gonadotropin genes in αT3-1 and LβT-2 cells. The total RNA was extracted and reverse transcribed from both cell lines, and RT-PCR analysis was performed using specific primers for each mouse gene as listed in Table 1. Left and right three lanes except both ends represent the expressions of three subunits of gonadotropin genes in αT3-1 and those in LβT-2 cells, respectively. DNA size markers are shown in both ends.

a double-fluorescent staining to precisely position the localization of UCH-L1 protein in the anterior pituitary gland. As shown in Fig. 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 cytoplasm of many FSH-, LH- and PRL-producing cells (Fig. 3c, d and f), while not in those of TSH-, ACTH- and GH-producing cells (Fig. 3a, b, e). In addition, we 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. 3g).

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

We 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, we 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. 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. 4i and j). Although a modest number of FSH-, LH- and PRL-expressing cells were observed in WT mice (Fig. 4c, e and g), to our surprise, obviously decreased number of FSH-, LH- and PRL-expressing cells were observed in *gad* mice compared to those in WT mice (Fig. 4d, f and h).

Expressions of UCH-L1 and other UCHs in gonadotrope cell lines

The data from *gad* mice suggested that UCH-L1 play an important role in FSH-, LH- and PRL-expressing cells. So, we examined also whether gonadotropes express UCH-L1 or not using gonadotrophic cultured cell lines αT3-1 and LβT-2 [1, 24]. aT3-1 and LβT-2 cells have been considered immature and mature types of gonadotropes, respectively [5, 24], which was supported by our data that LβT-2 cells only expressed *Fshb* and *Lhb* subunits gene in accordance with previous studies (Fig. 5). We examined both mRNA and protein expression levels of UCH-L1 in these two cell lines. The mRNA expression of *Uchl1* in αT3-1 cells was much higher than that in LβT-2 cells, with a statistical significance ($P<0.05$, Fig. 6A). However, this difference was not seen in the protein levels (Fig. 6B). Furthermore, semi-quantitative RT-PCR analyses of other UCH isozymes were also performed in these two cell lines. Although the expression levels of *Uchl4* and *Uchl5* were almost comparable between two cell lines, expression level of *Uchl3* in LβT-2 cells was significantly higher than that in aT3-1 cells, approximately 2.4-fold (Fig. 6A). However, the difference was not observed by Western blot analyses, in which the expression level of UCH-L3 protein was almost the same between two cell lines (Fig. 6B). Subsequently, we examined the distribution of UCH-L1 in these cell lines. As shown in Fig. 7, the localization of UCH-L1 exhibited a similar pattern between αT3-1 and LβT-2 cells, in which UCH-L1 was expressed throughout the whole cells, with bright fluorescence in the cytoplasm and a fractionally weak fluorescence in the nucleus.

Discussion

The ubiquitin-mediated protein degradation pathway is essential for eukaryotes and modulates many cellular processes [6]. The proteins which are targeted for proteolysis are labeled with polyubiquitin chains and eventually degraded by the 26S proteasome [30]. After degradation of target proteins, DUBs regenerate

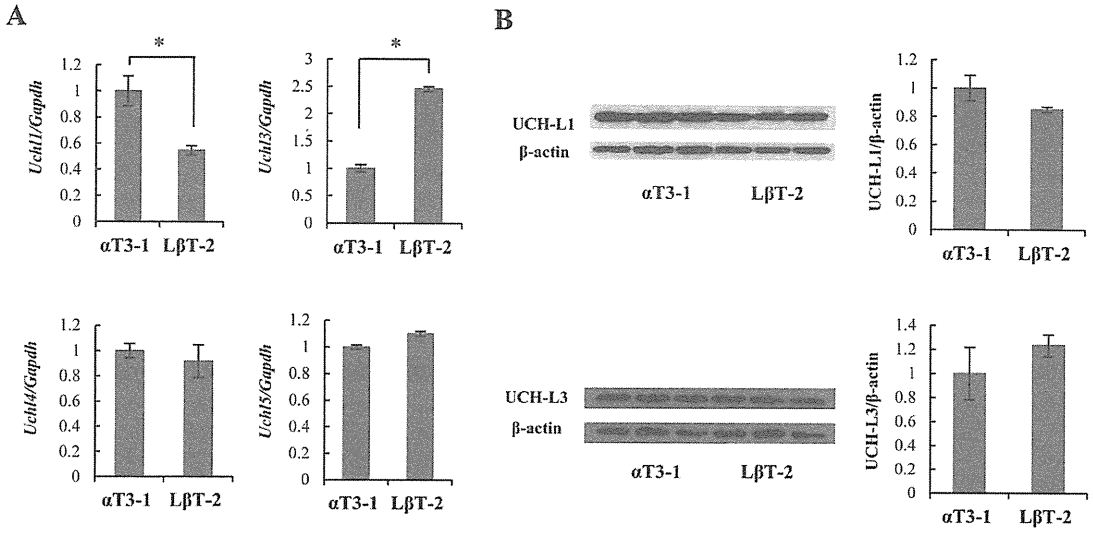

Fig. 6. The expressions of UCH-L1 and other UCHs in αT3-1 and LβT-2 cells. A: Semi-quantitative RT-PCR analyses of *Uchl1* and other UCH isozymes in αT3-1 and LβT-2 cells. The total RNA was extracted from these cells, and RT-PCR analysis was performed using specific primers as listed in Table 1. The graphs represent the averaged band intensities of UCHs with SEM, normalized with *Gapdh*. Statistical analysis was conducted using Student's *t*-test (**P*<0.05). B: Protein expression of UCH-L1 and UCH-L3 in αT3-1 and LβT-2 cells. αT3-1 and LβT-2 cell lysates were examined by Western blot on 12.5% gel. β-actin was used as a control. The graphs represent the averaged band intensities of UCH-L1 and UCH-L3 with SEM, normalized with β-actin. Statistical analysis was conducted using Student's *t*-test.

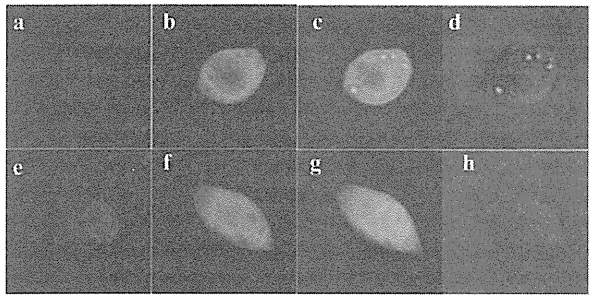

Fig. 7. The localization of UCH-L1 protein in αT3-1 and LβT-2 cells. To examine the localization of UCH-L1 protein in αT3-1 (upper panels) and LβT-2 cells (lower panels), immunofluorescent staining of UCH-L1 was conducted. TO-PRO-3 was used to visualize the nuclei (a, e). UCH-L1 (b, f), the merged (c, g) and transparent images (d, h) are presented. Images were photographed using a Zeiss LSM 510 confocal microscope.

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 [13, 29, 34]. 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 [14, 16, 26, 38, 39]. 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 [2]. In the present study, we 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 [32, 34]. 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, we have shown that the majority of the anterior pituitary cells was immunopositive 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, we 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 [9, 10]. 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 hormone 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 [37]. 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 [25]. Our 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 [18, 27]. Furthermore, overexpression of UCH-L1 caused the inhibition of spermatogenesis, eventually leading to male infertility [31]. 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 the present study, we 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 [36].

We 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. We 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 [19]. 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 [11]. However, the enhancement of UCH-L1 was dependent on interaction between UCH-L1 and CDKs, but not on its hydrolase activity. This also urges us to figure out how UCH-L1 functions in the anterior pituitary cells.

Gonadotropes synthesize and secrete FSH and LH, which are critical to both testis and ovary. We have a special interest in the effect of UCH-L1 on these cells. However, the pituitary gland of mice is small and this type of cells constitute approximately 10% of the anterior pituitary cell populations [8, 38]. It is not so easy to examine the role of UCH-L1 in gonadotropes in the pituitary gland. As an alternative approach, αT3-1 and LβT-2 cells, two immortalized cell lines established from the pituitary glands, were examined [1, 35]. UCH-L1 was found to be expressed in both nuclei and cytoplasm in these cell lines, which was consistent with our results *in vivo*. There are two hypotheses for the decrease in the number of gonadotropes in the pituitary gland of *gad* mice: 1) decrease in cell numbers by apoptosis; 2) failure to synthesize FSH or LH. αT3-1 cells are considered to represent immature type of gonadotropes and do not express β-subunits of gonadotropin. We detected a relatively comparable level of UCH-L1 in αT3-1 cells to that of LβT-2 cells, which might exclude a direct relevance between UCH-L1 and β-subunit expressions. However, some reports pointed out that the failure of synthesizing hormones in αT3-1 cells might be in part due to transcriptional suppressions [20]. Anyway, LβT-2 cells would be a useful model to study the function of UCH-L1 in gonadotropes and provide us an opportunity to examine the role of UCH-L1 in hormone production in gonadotropes using UCH-L1-specific inhibitor or RNAi technique in the future. In addition, we could examine whether UCH-L1 colocalized with FSH or LH in gonadotrope cell lines after GnRH stimulation as in mice (Fig. 3).

UCH-L1 and UCH-L3 are two predominant isozymes in mammals. These two isozymes are believed to have overlapping and reciprocal functions. Relative to *gad* mice, UCH-L1/UCH-L3 double knockout mice display a more severe axonal and cell body degeneration of the gracile tract [15]. On the other hand, UCH-L1 is considered as a pro-apoptotic regulator, while UCH-L3 is thought to be anti-apoptotic in a cryptorchid injury in

the testis [17]. Furthermore, our previous study revealed that UCH-L1 and UCH-L3 might play distinct roles in spermatogenesis, in which UCH-L1 was mainly expressed in spermatogonia, while the expression of UCH-L3 was predominantly detected in spermatocytes and spermatids [16]. As mentioned above, αT3-1 and LβT-2 cells are considered to represent immature and mature types of gonadotropes. In the present study, we have shown distinct mRNA expressions of *Uchl1* and *Uchl3* in these cell lines, although the protein expression levels of these two isozymes did not show a significant difference. This might reflect their different requirements during development of gonadotropes.

In conclusion, we 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.

Acknowledgements

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