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Feasibility analysis of ACTH adenoma model in USP8$^{-/-}$ mice

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Abstract

Introduction: Patients with adrenocorticotropic hormone (ACTH)-secreting pituitary tumours (35% to 60%) present with somatic mutations in the USP8 gene. USP8 mutations lead to enhanced deubiquitination of the epidermal growth factor receptor (EGFR) and result in an imbalance in EGFR signalling, accompanied by excessive activation of ACTH production and cell growth. USP8 emerged as a novel and exciting candidate gene for Cushing's disease.

Material and methods: In this study, USP8 mutant mouse models (USP8$^{+/-}$ and USP8$^{-/-}$) were established, their phenotypes were analysed and identified, biochemical indexes were detected, pituitary and adrenal tissue specimens were taken for HE staining and immunohistochemical identification of hormones, and the differences between the 2 groups of mutant mice and wild type mice were analysed and compared.

Results: Compared with the control group (wild type), immunofluorescence assay results for USP8$^{+/-}$ mice and USP8$^{-/-}$ mice showed increased pituitary ACTH expression, which was statistically different ($p < 0.05$), and there were no significant differences in body weight, plasma ACTH, 24-hour urinary free cortisol, and immunohistochemical results. Higher blood glucose in USP8$^{-/-}$ mice than in USP8$^{+/+}$ mice was observed. The heart rates of USP8$^{-/-}$ mice were higher than those of USP8$^{+/-}$ mice and USP8$^{+/+}$ mice. HE staining and tissue fibre staining were done, and no significant pathological changes were seen in the 3 groups of pituitary and adrenal tissues.

Conclusion: USP8 knockout mice have the potential to form an animal model of ACTH adenoma. **(Endokrynol Pol 2023; 74 (2): 181–189)**

Key words: *USP8; Cushing's disease; animal model; ACTH adenoma*

Introduction

Cushing disease (CD) is a serious disorder characterized by hypercortisolism, which in most cases is due to excessive secretion of pituitary adrenocorticotropic hormone (ACTH) [1]. Chronic exposure to excess glucocorticoids can lead to multisystem complications such as hypertension, diabetes, dyslipidaemia, osteoporosis, infections, cardiovascular disease, and psychiatric disorders [2], resulting in increased mortality and reduced quality of life [3, 4].

Ubiquitin-specific proteases (USPs) are deubiquitinating enzymes (DUBs) that play a key regulatory role in many therapeutically relevant processes in cancer [5, 6]. In 2015 Reincke highlighted the role of USP8 in pituitary tumourigenesis by identifying functionally acquired somatic mutations in the USP8 gene, which shows a specific specificity for pro-adrenocorticotropic tumours [7]. In this case, USP8 mutations led to enhanced deubiquitination of the epidermal growth factor receptor (EGFR) resulting in an imbalance in EGFR signalling accompanied by excessive activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2), proopiomelanocortin (POMC) transcription, ACTH production, and cell growth [8]. USP8 emerged as a novel and exciting candidate gene for Cushing's disease; however, mouse models linking USP8 mutations to Cushing's disease have not been reported in any article.

In this paper, we constructed USP8 heterozygous knockout (USP8 $^{+/-}$) mice as well as USP8 homozygous knockout (USP8$^{-/-}$) mice. Although the USP8$^{+/-}$ mice and USP8$^{-/-}$ mice did not perform significantly differently from wild-type (WT) controls in body weight, plasma ACTH, 24-hour urinary free cortisol, and organ pathology section analysis, USP8$^{+/-}$ mice and USP8$^{-/-}$ mice had significantly higher pituitary ACTH expression, demonstrating a specificity of this gene mutation with respect to adrenocorticotropic tumours. Thus, USP8 knockout mice are likely to be a candidate model for Cushing's disease.

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Material and methods

Animal sources

SPF grade C57BL/6 mice, 30 mice, weighing 18–22 g, purchased from Saiye Model Biological Research Centre (Taicang). License number: SCXK(Su)2018-0003. The animal use protocol and animal handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University. Animals were raised in separate cages and had free access to standard rodent food and water. Room temperature was maintained at 25~26 , humidity was maintained at 50~60%, and the animals were housed in a 12-h day/night cycle lighting environment.

Constructing USP8 knockout mice

Design sgRNA according to gene sequence, construct gene targeting vector pesSpCas9-sgRNA and screen the sgRNA with higher activity. Design primers containing T7 promoter to amplify eSpCas9 and the aforementioned active sgRNA respectively and recover and purify them. An in vitro transcription kit transcribed eSpCas9 and sgRNA into mRNA. Meanwhile, the donor sequence was determined according to the target site, and donor DNA oligos was synthesized in vitro. Then the above reagents were co-injected into the fertilized eggs of mice, and the F0-generation mice were obtained after transplantation of pseudopregnant mice. F0 mice were tail clipped at about 2 weeks, and the kit extracted genomic DNA, designed specific primers for polymerase chain reaction (PCR) amplification of DNA fragments near the target site, and the PCR products were sent for sequencing to obtain positive mice.

Genotype identification

After 20 days of single-cage rearing of fertilized ovum-transplanted pseudopregnant female mice, the suckling mice were observed at birth. The genomic DNA was extracted from 1 cm of tail-tip tissue of F0-generation mice at 2 weeks of age, and the primers USP8-F and USP8-R (Tab. 1 for primer sequences) were used for PCR amplification in the target region of the USP8 gene, and the PCR products were sequenced to identify mouse genotypes.

Blood glucose monitoring and blood pressure measurement methods

We detected the blood glucose concentrations of mice by tail vein blood collection method, and we measured the blood pressure of mice by optical volume pulse method through the correspondence between the pressurization sensor and pulse wave indirectly and non-invasively to obtain the measurement of the blood pressure of the mouse – heart rate, systolic blood pressure, and diastolic blood pressure. We automatically determined whether the mouse was in a stable state through the pulse wave and then started measuring.

Plasma ACTH concentration measurement

Blood was collected for ACTH measurement between 12:00 noon and 2:00 p.m. Plasma was separated by centrifugation at 800 g for 10 min at 4°C and stored at –20°C prior to analysis. Plasma ACTH concentrations were quantified using an ELISA kit.

Table 1. *Primers used in this study*

Primer	Primer sequence (5′→3′)
Mouse Usp8-F	GTCTGTGCTTAGCAAATTCAAGGCC
Mouse Usp8-R	GGGCATGGTACTGGGAAAGTGCT

Collection and measurement of 24-hour urinary free cortisol

Urine was collected from 12-week-old mice in a collection tube for 24 hours. Sample volumes were recorded, and each sample was centrifuged at 800 g for 10 min at 4°C, and aliquots were stored at –20°C prior to analysis. Urinary free cortisol concentrations were quantified using an ELISA kit.

Haematoxylin and eosin (H&E) staining

The pituitary and adrenal tissues of WT, USP8$^{-/-}$ mice and USP8$^{+/-}$ mice of the same age were fixed in 4% paraformaldehyde for 24 hours, rinsed in running water for 24 hours, dehydrated in anhydrous ethanol for 3 hours, and transparentised in xylene for 1 hours. The tissues were embedded in paraffin and cut into 5-μm-thick sections with a paraffin microtome (Leica, Germany) for H&E. They were sealed with neutral gum and observed microscopically.

Reticulate fibre staining

Tissue sections were dewaxed, oxidized by dropping into 0.3% hydrogen peroxide solution for 2–3 min, washed twice with distilled water, underwent mordanting with 2% ferric ammonium sulphate solution for 5–10 min, were washed with distilled water, exposed to Gomori silver ammonia solution for 3 min, rinsed with distilled water, reduced by dropping into 4% neutral formaldehyde solution for 1 min, washed with distilled water, toned with 0.2% aqueous gold chloride solution for a few seconds, observed under a microscope, washed with distilled water, re-stained nucleus solid red for 3 min, dehydrated with ethanol gradient, transparentised with xylene, and sealed with neutral gum.

Immunofluorescence staining

The tissue sections were rewarmed for 30 min, closed, and incubated overnight at 4′ with primary antibody (ACTH, 1:500), the fluorescent secondary antibody (fluorescein isothiocyanate, 1:200) was added to the sections protected from light, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and sealed with glycerol. Analysis was performed with Image pro 5.0, and the mean optical density of positive expression was measured.

Statistical analysis

Data were statistically analysed using GraphPad Prism 8. 4. 0 and IBM SPSS Statistics 26, and all data in the experiments were expressed as mean ± standard deviation ($x \pm s$). The independent samples t-test was used for comparison between 2 groups, and one-way ANOVA was used for comparison of multiple groups. If $p < 0.05$, it indicated that the difference was statistically significant.

Results

Construction of USP8 knockout mice

Genomic DNA was extracted by cutting mouse tails, PCR amplification was performed using primers USP8-F and USP8-R (Tab. 1 for primer sequences), and the PCR products were sequenced. The sequencing analysis was compared with the genome sequence of WT mice. Among the 36 USP8 knockout mice obtained, there were 13 USP8$^{-/-}$ mice and 23 USP8$^{+/-}$ mice, and the original sequencing of the mice were compared with peak plots (Fig. 1A).

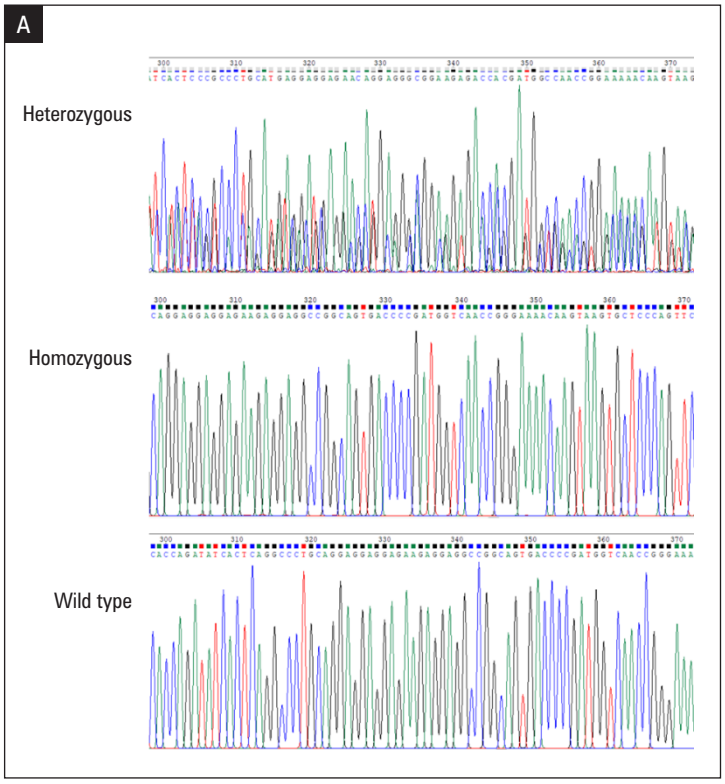

Figure 1. *Genotype sequencing of USP8 mutant mice.* **A.** *USP8$^{-/+}$, USP8$^{-/-}$ mice, USP8$^{+/+}$ mice original sequencing sequence comparison with peak plots (n = 13 mice per group)*

Phenotypic analysis of USP8 mutant mice and wild-type mice

The USP8$^{+/-}$, USP8$^{-/-}$, and USP8$^{+/+}$ mice had the same appearance and fur colour distribution phenotype, no significant differences and no abnormal changes in appearance, and good diet, growth, and mental activity were observed. The mice were divided into 3 groups according to their genotypes: USP8$^{+/+}$ mice were divided into wild type, USP8$^{+/-}$ mice were divided into heterozygous, and USP8$^{-/-}$ mice were divided into homozygous groups. Body weights were measured weekly from the second week of life until the end of the 40th week for the 3 groups of mice, and the data were recorded and statistically analysed to show no statistical difference between the 3 groups (Fig. 2A). The heart rates of USP8$^{-/-}$ mice were higher than those of USP8$^{+/-}$ mice and USP8$^{+/+}$ mice (Fig. 2B). We measured blood pressure in mice using a mouse smart noninvasive sphygmomanometer, and the differences in systolic and diastolic blood pressure were not statistically different in each group (Fig. 2C).

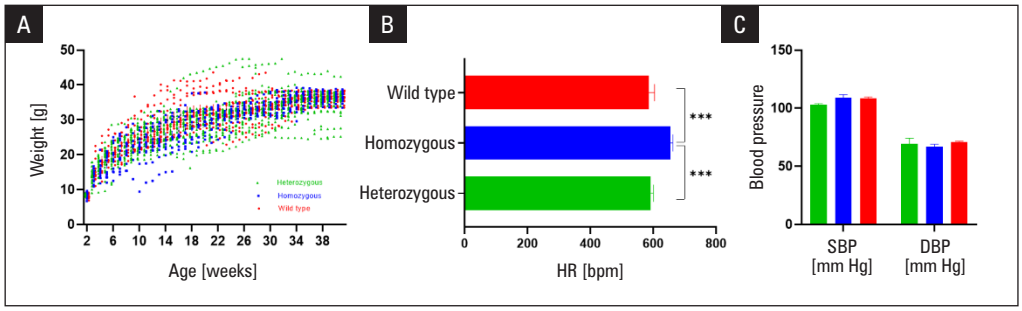

Figure 2. *Phenotypic analysis of USP8 mutant mice.* **A.** *The weight of mice in the 3 groups was measured weekly from the second week of birth to the 40th week (F = 1.016, p = 0.3654, n = 13 mice per group);* **BC.** *The heart rate (F = 15.92, p < 0.0001, p = 0.0002, n = 13 mice per group) and blood pressure of mice in the 3 groups were measured every other week from week 2 to week 40. Data are expressed as means ± standard error of mean (SEM). ***p < 0.001*

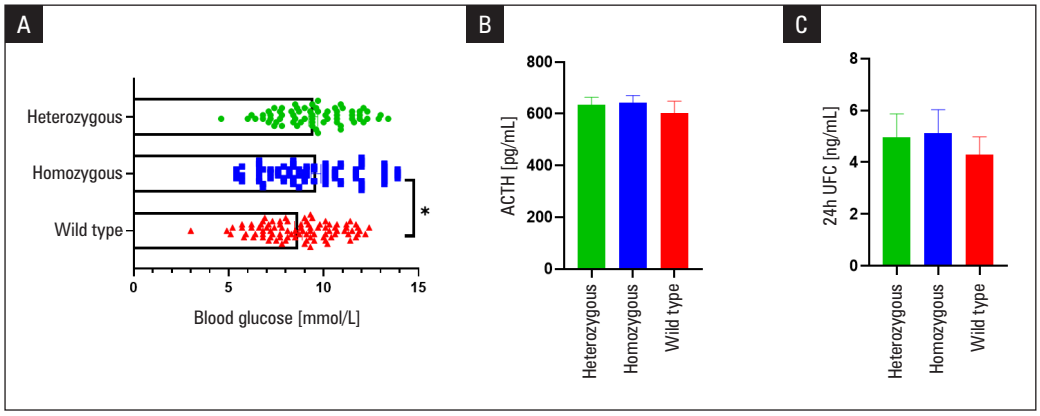

Figure 3. *Biochemical indices of USP8 mutant mice and wild-type mice.* **A.** *The blood glucose of mice in the 3 groups was measured every other week from week 2 to week 40 ($F = 4.700$, $p = 0.0123$, $n = 10$ mice per group);* **B.** *Plasma adrenocorticotropic hormone (ACTH) content of 3 groups of mice ($F = 0.3646$, $p = 0.6978$, $n = 10$);* **C.** *24-hour urinary free cortisol (UFC) levels of the 3 groups mice ($F = 0.2706$, $p = 0.7650$, $n = 10$). Data are shown as means ± standard error of mean (SEM). *$p < 0.05$*

Higher blood glucose in USP8$^{-/-}$ mice than in USP8$^{+/+}$ mice

For the 3 groups of mice, the blood glucose was measured every other week starting from the 2nd week, and at the end of the 40th week the statistical results showed that the blood glucose of USP8$^{-/-}$ mice was higher than that of USP8$^{+/+}$ mice, and the difference was statistically significant (Fig. 3A). It is worth noting that the blood glucose of 3 groups of mice was within the normal range without reaching the standard of diabetes.

USP8 knockdown has no significant effect on plasma ACTH and urinary free cortisol in mice

The UFC reflects the combined tissue exposure to free cortisol over a 24-hour period and therefore provides a unique perspective on glucocorticoid physiology that differs from the dexamethasone suppression test [9]. We collected 24-hour urine from mice and measured free cortisol levels, and the differences between the 3 groups were not statistically significant (Fig. 3C). Plasma was taken from the 3 groups of mice for ACTH hormone assay, and the differences between the 3 groups were also not statistically significant (Fig. 3B).

Pathological analysis of tissues from USP8 mutant mice vs. wild-type mice

At 40 weeks, the mice were euthanised, and it was initially found by anatomy that there was no significant difference in the colour of each tissue morphology between USP8$^{-/-}$ mice and USP8$^{+/-}$ mice compared with USP8$^{+/+}$ mice, and no histopathological condition appeared (Fig. 4A), while the effect of USP8$^{-/-}$ knockout on the tissue physiological function of the mice needs further study. The pituitary and adrenal tissues of mice were taken, and H&E staining and tissue fibre staining were done, and no significant pathological changes were observed in the pituitary glands and adrenal glands in all 3 groups (Fig. 4BC).

POMC and ACTH expression in pituitary and adrenal glands of USP8 mutant mice vs. wild-type mice

Frozen sections of pituitary and adrenal tissue were subsequently subjected to POMC immunohistochemistry, and the differences between the 3 groups were not statistically significant when analysed using image Image-Pro Plus software (Fig. 5A). ACTH immunofluorescence results showed that both USP8$^{-/-}$ mice and USP8$^{+/-}$ mice had higher ACTH expression in the pituitary or adrenal glands than USP8$^{+/+}$ mice, and the differences were statistically significant (Fig. 5BC). Western blot results of ACTH expression in pituitary tissues of mice in the 3 groups were consistent with immunofluorescence results (Fig. 5D).

Discussion

The finding that somatic mutations in the USP8 gene are presented in patients (35% to 60%) with ACTH-secreting pituitary tumours represents a further step in our understanding of Cushing's disease and a new understanding of the ubiquitination/de-ubiquitination system in the pathophysiology of ACTH adenomas [7, 10]. The balance between intracellular ubiquitination and deubiquitination determines which proteins are degraded, stored, and recycled. USPs and DUBs are indeed key regulators of the stable levels of various proteins involved in cell cycle progression, apoptosis, and DNA damage repair [11, 12]. USP8 mutant

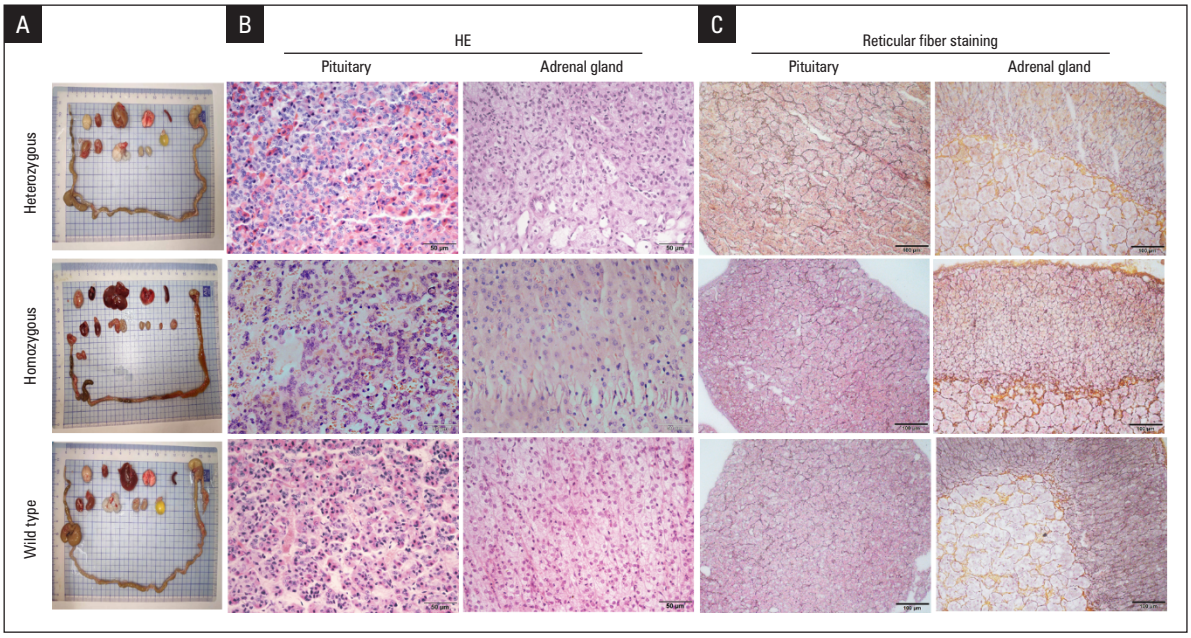

Figure 4. *Histopathological analysis of USP8 mutant mice versus wild type mice.* **A.** *There was no obvious difference in the morphology and colour of the tissues in the 3 groups and no pathological changes occurred;* **BC.** *Haematoxylin and eosin (H&E) staining and tissue fibre staining were performed on pituitary and adrenal gland tissues of mice in the 3 groups and none had obvious pathology. n = 3 mice per group*

pro-adrenocorticotropic adenomas exhibit a more "typical" pro-adrenocorticotropic phenotype with marked pro-adrenocorticotropic synthesis and secretion, and genes associated with proteasomal degradation mechanisms have been shown to be repressed in USP8 mutant adenomas [13]. Overexpression of the USP8 G664R variant in AtT-20 cells can increase ACTH secretion and cell proliferation by affecting USP8/14-3-3 protein binding, leading to USP8 protein hydrolytic cleavage [14]. The USP8 mutation results in a significant loss of physiological inhibition of USP8 deubiquitinase activity by the 14-3-3 protein, leading to increased recycling of its substrates, such as the receptor tyrosine kinase epidermal growth factor receptor (EGFR) [7, 15], which promotes cell proliferation and ACTH secretion due to sustained EGFR signalling [16]. USP8 may be a potential target for the treatment of Cushing's disease (CD), and inhibition of USP8 inhibits adrenocorticotropic hormone secretion and ACTH adenoma cell growth in AtT20 cells [17–20]. However, studies combining USP8 mutations with animal models have not been reported.

The establishment of an animal model of pituitary adenoma is essential for the in-depth study of the mechanism of pituitary adenoma development [21]. HeIseth et al. established the first transgenic animal model of ACTH adenoma by microinjecting an early promoter gene attached to the cDNA encoding the polyomavirus large T antigen (PyLT) into the spermatogonial nucleus of fertilized eggs of mice [22, 23]. Stenzel *et al.* successfully produced a mouse model of Cushing's disease by transgenic technique to overexpress CRH [24]. However, the pituitary tumour could not be observed by the naked eye in the early stage of the model constructed by this technique, and the specificity was not high as well as the overexpression caused by random insertion made the mouse phenotype unstable. In 2014, Bentley *et al.* established a mouse model of Cushing's syndrome by inducing CRH promoter mutation by ethylnitrosourea (ENU), which caused CRH overexpression in mice [25]. However, the induced models are not very stable because of differences in inducers and animal quality (especially genetic background), and the mechanisms of genetic variation in tumorigenesis vary. Liu *et al.* established a zebrafish ACTH adenoma model for pituitary POMC using transgenic techniques to express the Pttg gene [26]. However, the evolutionary difference between zebrafish and humans makes it difficult to realistically mimic the development of human ACTH adenomas.

Cushing's disease has an exceptional clinical presentation with multiple comorbidities, mainly including systemic arterial hypertension, as well as visceral obesity, impaired glucose tolerance, and dyslipidaemia, constituting a metabolic syndrome [27–29]. Therefore, we measured the body weight and blood glucose of mice in this study, in which there was no significant difference between the body weight of USP8 mutant mice

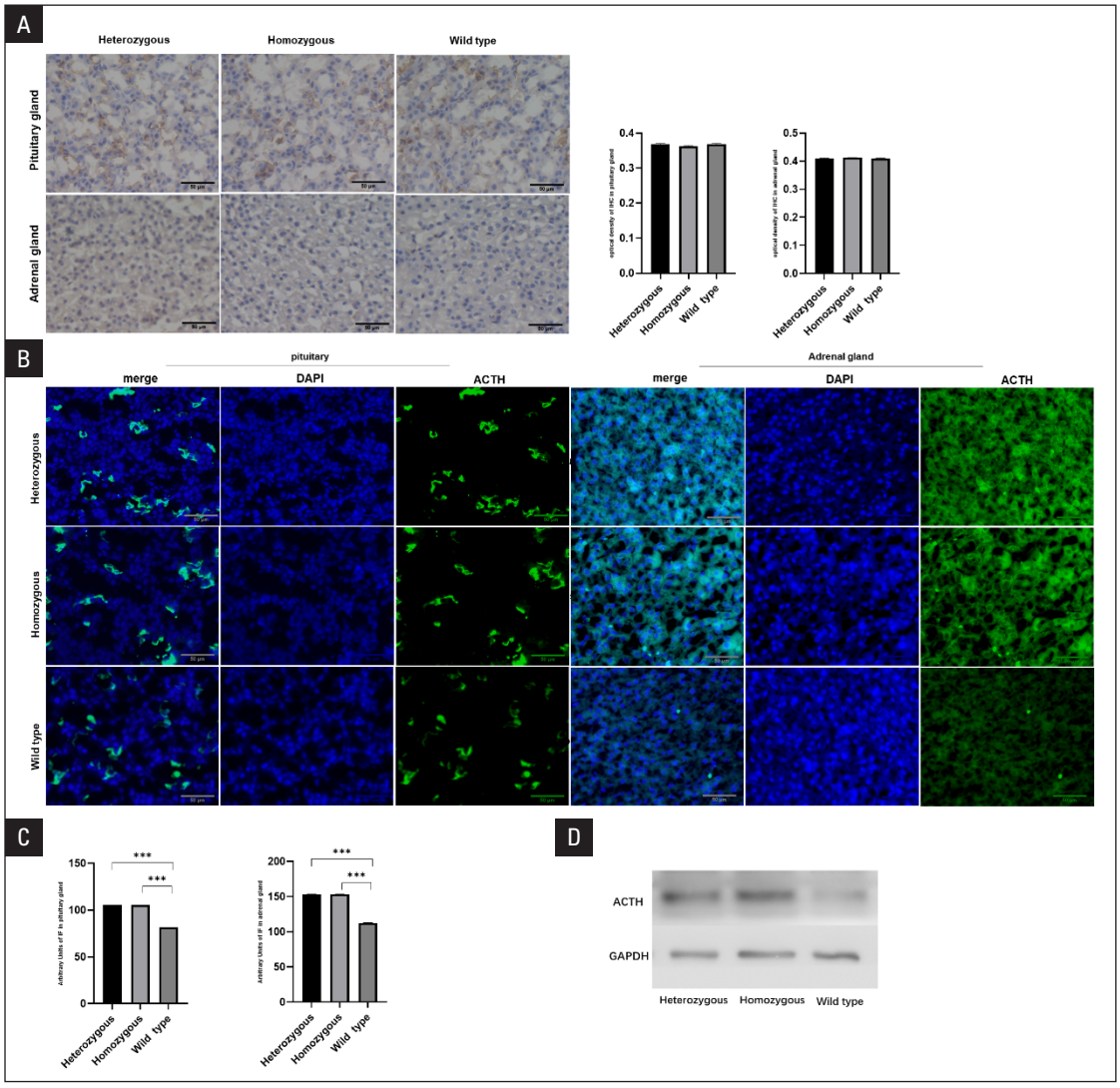

Figure 5. *Proopiomelanocortin (POMC) and adrenocorticotropic hormone (ACTH) expression in USP8 mutant mice versus wild-type mice.* **A.** *POMC immunohistochemistry was performed on the frozen sections of pituitary and adrenal tissues of the 3 groups of mice, and there was no difference between the 3 groups ($p = 0.0903$, $p = 0.4702$, $n = 3$);* **BC.** *ACTH immunofluorescence was performed on pituitary and adrenal gland tissues of mice in the 3 groups, and the fluorescence intensity of the wild type group was significantly lower than that of the other 2 groups ($p < 0.001$, $n = 3$);* **D.** *Western blot results of ACTH expression in pituitary tissues of mice in the 3 groups. Data are shown as mean ± standard error of mean (SEM) for each group. ***$p < 0.001$*

and wild mice, and USP8 knockout had no significant effect on the body weight of mice. Notably, Wei Bia et al. found that increased expression of USP8 resulted in reduced weight loss in mice with a sepsis-associated encephalopathy (SAE) model [30]. Clinical studies have shown no significant relationship between USP8 and body weight in patients with advanced non-small cell lung cancer (NSCLC) [31]. Because the regulation of body weight and composition is complex and influenced by genetic structure, environment, and their interactions [32], whether USP8 mutations have a regulatory effect on body weight and composition in mice needs further investigation. It has been reported in the literature that the diabetes gene Clec16a encodes an E3 ligase that promotes nondegradative ubiquitin splices to direct its mitochondrial autophagic effects and stabilize the Clec16a-Nrdp1-USP8 complex [33]. Therefore, knockdown of USP8 may affect the stability of the Clec16a-Nrdp1-USP8 complex and thus inhibit insulin secretion and increase blood glucose, which could explain why blood glucose is higher in USP8$^{-/-}$ mice than in heterozygous and wild-type mice.

Indeed, in all species, including humans, there is a strong inverse relationship between heart rate and lifespan [34, 35]. Higher heart rate is associated with increased mortality in mice [36]. Heart rate is an independent risk factor for cardiovascular diseases, especially myocardial ischaemia and heart failure

[37–40]. Because the results show that USP8$^{-/-}$ mice have a higher heart rate than wild-type mice, USP8 mutant mice may have a higher risk of cardiovascular disease than wild-type mice. No cardiovascular disease was found in the USP8 mice, and the blood pressure of the 3 groups of mice did not differ significantly and did not meet the criteria for hypertension in mice [41]. The 3 groups of mice did not differ significantly in blood pressure and did not meet the criteria for hypertension in mice. This suggests that knockdown of USP8 mice increases the heart rate but has little effect on the blood pressure in mice. The histopathological findings of the 3 groups of mice, including HE staining and tissue fibre staining of the pituitary and adrenal glands, showed no lesions, and there were no significant differences in plasma ACTH and 24-hour urinary free cortisol. It is worth noting that the expression of ACTH in the pituitary of USP8 mutant mice was significantly higher than that of wild type mice. This indicates that the USP8 mutation can affect the expression of ACTH in the pituitary of mice and increase it.

In this case the ACTH expression was increased in the pituitary tissue of USP8 mutant mice, but not in the blood. We think that there are several possibilities for this mechanism. Firstly, Kovacs et al. [42] observed that silent corticotroph adenoma (SCA) cells have a high number of lysosomes in the cytoplasm and show fusion of these lysosomes with secretory granules as shown by electron microscopy, leading to the hypothesis that ACTH is destroyed before it can be released. We speculated that the pituitary cells secreting ACTH in USP8 mutant mice might contain more lysosomes, leading to the destruction of ACTH before release and ultimately no elevation of ACTH in the blood. Then, given the fact that there were no significant differences in POMC expression between USP8 mutant and wild-type mice, we suggested that the function or expression of prohormone convertase (PC) 1/3, a POMC-processing enzyme, may be disturbed in USP8 mutant mice. PC1/3, encoded by the PCSK1 gene, is involved in the processing of POMC into mature and biologically active ACTH [43]. Tateno *et al.* [44] and Jahangiri *et al.* [45] observed that the expression level of PC 1/3 in CDs was 15 times and 30 times that of SCAs, respectively, and low PCSK 1 gene was also observed, further confirming that the defect of PC 1/3 is related to the transformation of POMC into mature bioactive ACTH [46]. Post-translational regulation of ACTH has also been studied [45–47], and we believe that the proconvertase PC2 may also be involved in this mechanism. Encoded by PCSK2, PC2 is an endoproteolytic enzyme responsible for the processing of biologically active ACTH into α-melanocyte-stimulating hormone and corticotropin-like intermediate lobe peptide in the intermediate lobe [48]. The USP8 gene may affect the expression of proconvertase PC2 to degrade ACTH in USP8 mutant mice, which partly explains the increased expression of ACTH in pituitary tissue, but not in blood. In addition, peptidylglycine α-amidating monooxygenase (PAM) and carboxypeptidase E (CPE), both involved in the post-translational regulation of ACTH, might lead to the inability to secrete ACTH in blood [46]. At the last microRNAs (miRNAs) have also been proposed to play a potential role in this mechanism [49]. García-Martínez [42] observed significantly higher levels of miR-200a and miR-103 in SCAs than in CDs, which suggests that these miRNAs may be the reason why ACTH in blood of SCAs does not increase [47]. We ponder that these miRNAs might be related to USP8 in some way, and the increased expression in USP8 mutant mice eventually prevented the increase of ACTH in the blood of USP8 mutant mice. Therefore, the exact mechanism by which the USP8 mutation makes mice increase ACTH expression without causing Cushing's disease needs to be further investigated. Other mutations in the glucocorticoid receptor NR3C1, BRAF oncogene, deubiquitinase USP48, and TP53 have been identified in whole exome sequencing studies of Cushing's disease cases, but at much lower incidence rates [50, 51]. Because tumour formation, growth, and invasion are also influenced by DNA methylation, histone modifications, and epigenetic mechanisms mediated by noncoding RNAs such as microRNA, long noncoding RNA, and cyclic RNA [52], the specific mechanism of knocking out the USP8 gene alone may not directly construct an animal model of Cushing's disease, and further studies are needed.

In this study, we established USP8 mutant mouse models of USP8$^{+/-}$ mice and USP8$^{-/-}$ mice, and confirmed the increased expression of ACTH in USP8 mutant mice, providing a reference for further investigation of the regulatory mechanism of USP8 on the occurrence of Cushing's disease in the future.

Conflict of interests

The authors state that there are no conflicts of interest to disclose.

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Not applicable.

Ethics approval

Ethical approval was obtained from the Ethics Committee of the Zhongshan School of Medicine of Sun Yat-sen University.

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