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A Histopathological Study of Multi-hormone Producing Proliferative Lesions in Estrogen-induced Rat Pituitary Prolactinoma

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Rats with estrogen-induced prolactin-producing pituitary adenoma (E2-PRLoma) have been employed as an animal model of human PRL-producing pituitary adenoma in a large number of studies. Presently, we found that long-term administration of estrogen to SD rats resulted in the development of E2-PRLomas, some of which included multi-hormone producing nodules. We herein report results of histopathological analyses of these lesions. PRLoma models were created in female SD rats by 22 weeks or longer administration of a controlled-release preparation of estradiol at a dose of 10 mg/kg/2 weeks. Ten of the 11 PRLoma model rats had proliferative nodular lesions composed of large eosinophilic cells like gonadotrophs inside the PRLoma. These lesions were positive for PRL, TSHβ, and α subunits and were negative for GH, LHβ, ACTH, and S-100. Double immunostaining revealed that these large eosinophilic cells showed coexpression of PRL and TSHβ, PRL and α subunits, and TSHβ and α subunits. Those results clarified that long-term estrogen administration to female SD rats induced multi-hormone producing neoplastic pituitary nodules that expressed PRL, TSHβ, and α subunits. We studied these neoplastic nodules obtained by laser microdissection to acquire findings similar to those of the immunohistochemical analysis. We consider that this animal model is useful for pathogenesis analyses and therapeutic agent development concerning human multi-hormone producing pituitary adenomas.

Key words: prolactinoma, multi-hormone producing adenoma, estrogen, laser capture microdissection

I. Introduction

The pituitary cells can be classified into three cell lineages: the growth hormone (GH)-prolactin (PRL)-thyroid-stimulating hormone (TSH) (GH-PRL-TSH) cell lineage, the proopiomelanocortin (precursor of adrenocorticotropic hormone, ACTH) lineage, and the gonadotropin (luteinizing hormone/follicle-stimulating hormone; LH/FSH) lineage [30]. Human pituitary adenomas are either clinically functioning or non-functioning [29]. The former includes GH secreting adenomas (GHomas), PRL secreting adenomas (PRLomas), TSH secreting adenomas (TSHomas), ACTH secreting adenomas (ACTHomas), and FSH/LH secreting adenomas (FSHomas/LHomas). LHomas are rather rare [8, 20, 24]. More frequent are GHomas and PRLomas. PRLoma occurs with relatively greater frequency comprising 25 to 41% of pituitary adenomas and

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produces only PRL in principle [15]. On the other hand, the GH-producing adenoma (GHoma) is a mixed somatotroph-lactotroph adenoma containing GH-producing as well as PRL-producing cells [32]. Also, the mammosomatotroph adenoma contains cells that produce GH as well as PRL [14]. Moreover, pituitary adenomas such as GHoma and PRLoma occur in McCune-Albright syndrome as implicated by the *Gs* α gene [6]. The Carney complex implicated by the *Prkar1*α gene develops such pituitary adenomas as GHoma and PRLoma while all types of pituitary adenomas arise in MEN1 caused by the *Menin* gene [1]. Additionally, it is known that GHoma, PRLoma, and TSHoma occur in *PROP1* transgenic and *Prkar1*α knockout mice [4, 13]. Meanwhile, it has been assumed that the estrogen-induced prolactin-producing adenoma (E2-PRLoma), an experimental model used in the current study, produces only PRL [2, 21, 26].

We found for the first time in the current study that female SD rats incurred lesions of E2-PRLoma as well as neoplastic nodules comprised of large eosinophilic cells differing in morphology from conventional PRLoma cells. A pilot study strongly suggested that these neoplastic nodules were tumors that produced a variety of hormones. Consequently, we performed simple and double staining to study in detail hormone expressions in these neoplastic nodules. Moreover, we obtained separately multi-hormone producing nodules, non-nodules, and normal tissues by laser microdissection to analyze mRNA expressions of pituitary hormones in an attempt to exactly characterize hormone production by these neoplastic nodular lesions.

II. Materials and Methods

Animals

Female Crl:CD rats, 7 weeks old, were purchased from Charles River Co. (Kanagawa, Japan). Animals were randomly allocated to 3 groups based on body weight (n=5, 5 and 11). Two groups received 10 mg/kg estradiol (E2; estradiol dipropionate: Ovahormone Depot®, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) every other week intramuscularly for 14 (n=5) or 22–36 (n=11, long-term E2 administered group) weeks. The third group was injected with sesame oil (Sigma-Aldrich, Inc., St Louis, MO, USA) as a control (n=5). This experiment was approved by the Animal Experimentation Committee, Isehara campus (Tokai University, Kanagawa, Japan).

Tissue preparation

Animals were sacrificed following 14 or 22 to 36 weeks dosing with E2. Pituitary glands were collected, weighed and fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). The uterus and mammary gland, which are target tissues of estrogen, were collected and fixed overnight in Mildform 10N (Wako, Osaka, Japan) to check the effects of estrogen. For histological analysis, fixed pituitary glands were embedded in paraffin and cut into 4 μm sections, which were stained with hematoxylin and eosin (H&E) according to the standard method.

Immunohistochemistry

For immunohistochemical studies the following antibodies were used and diluted as indicated: anti-prolactin (PRL) antibody from the National Institute of Diabetes and Digestive and Kidney (NIDDK, Baltimore, MD, USA, 1:1000), anti-growth hormone (GH) antibody (NIDDK, 1:1000), anti-thyroid-stimulating hormone β (TSHβ) antibody (NIDDK, 1:1000), anti-glycoprotein hormone alpha-subunit (αSU) antibody (NIDDK, 1:1000), anti-luteinizing hormone (LHβ) antibody (NIDDK, 1:1000), anti-S-100 protein antibody (Dako, Carpinteria, CA, USA, 1:500), anti-adrenocorticotrophic hormone (ACTH) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100), anti-PCNA (Dako, Tokyo, Japan, 1:500), and anti-Ki67 (Leica Biosystems, Newcastle, UK, 1:100). Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase activities were blocked in 0.3% hydrogen peroxide in methanol for 30 min at room temperature. For PRL, GH, TSHβ, αSU, LHβ, S-100, PCNA and Ki67 staining, sections were boiled in a microwave for 10 min at 98°C in HistVT One® (pH 7.0; Nacalai Tesque, Kyoto, Japan) for antigen retrieval. Sections were then incubated in 10% normal goat serum (NGS) blocking buffer for 30 min at room temperature. Sections were incubated with primary antibodies for 1 hr at room temperature. After the primary antibody reaction, sections were incubated with secondary antibody using Histofine® Simple Stain MAX-PO MULTI (Nichirei Bioscience Inc., Tokyo, Japan) for 60 min at room temperature. The sections were visualized with 3'3-diaminobenzidine (DAB). A double-staining technique was performed to examine the possible co-localization of hormones. After DAB visualization, sections were incubated in glycine-HCl buffer (pH 2.2) to remove the immuno-complex. Sections were incubated with Histofine® Simple Stain AP for 1 hr at room temperature and visualized by Fast Blue RR Salt (Sigma Aldrich) and naphthol AS-BI phosphate disodium salt (Sigma Aldrich) in 0.05 M propanediol buffer (pH 9.8).

Laser microdissection and real-time quantitative RT-PCR (qRT-PCR)

4-μm thick sections were counterstained with toluidine blue. For normal pituitaries, non-nodular pituitaries, and nodular pituitaries, a laser microdissection assay was performed using a laser microdissection system (LM) (Carl Zeiss MicroImaging, Jena, Germany) coupled with real-time reverse transcriptase PCR [22]. Three tissue sections were collected by LM for mRNA analysis. It was possible to analyze mRNA levels of 20 genes collected from three tissue sections. Total RNA was extracted from dissected normal pituitary, non-nodular pituitary, and nodular pituitary sections using RNeasy FFPE Kit (QIAGEN, Hilden, Germany). For synthesis of first-strand cDNA, RNA was

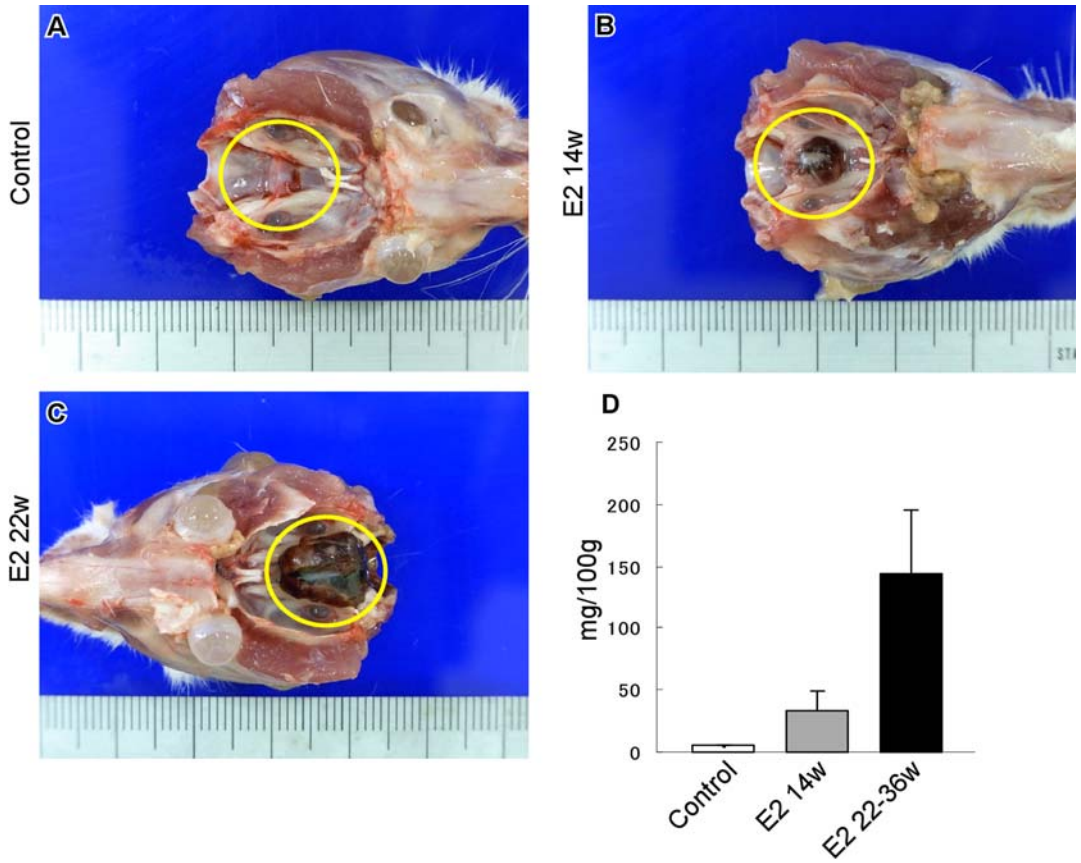

Fig. 1. Pituitary weights and gross abnormality. (**A, B, C**) Prodigious pituitaries were found in the E2 rat group. The E2 administration group showed a dusky-red swollen pituitary (area surrounded by a yellow circle). (**D**) Relative pituitary weights were calculated as mg/100g. E2 administration increased pituitary weights with time, with markedly increased pituitary weight with long-term E2 administration (22 to 36 weeks).

reverse transcribed by incubation with random primers and a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, and PRL, GH, TSHβ, αSU, and GAPDH were quantified using commercially available kits (TaqMan Gene Expression Assays Rn00561791_m1, Rn014955894_g1, Rn00565424_m1, Rn02532426_s1, and Rn99999916_s1, respectively; Applied Biosystems). These primer sets were designed to span one intron to allow identification of genomic contamination. The reaction protocol consisted of the following cycles: 95°C for 15 min, 95°C for 15 sec, and 60°C for 1 min for 50 cycles of PCR amplification on an Opticon 2 System (BioRad, Hercules, CA, USA). All data were analyzed on an Option monitor 3 (BioRad).

Statistical analysis

Values are expressed as means±SD. Differences were analyzed by Student's t-test, and statistical significance was considered when $P<0.05$.

III. Results

Creation of pituitary adenoma models by long-term estrogen administration and histopathological analysis of the pituitary

11 female SD rats (Crj:CD (SD) rats) were intramuscularly injected with an estradiol dipropionate preparation (5 mg Ovahormone Depot, Asuka Pharmaceutical Co.) at a dose of 10 mg/kg once per 2 weeks for a period of 22 to 36 weeks to create PRLoma models. If a rat's general condition worsened as manifested, for example, by decreased locomotor activity or lowered body temperature, it was sacrificed to be excluded from the study. Another 5 female SD rats were similarly injected with a mixture of benzyl benzoate and sesame oil to serve as controls. In addition, the pituitary glands from rats to which E2 was administered for 14 weeks were used for studies of conventional prolactin-producing pituitary adenomas. The macroscopic appearance of pituitary glands of an animal from each of the 3 groups as well as weight changes are shown in Figure 1. Increases in weight were as follows: 4.4±0.5 mg/100g in control rats, 33.3±15.5 mg/100g in 14-week E2 rats, and 144±51.6 mg/100g in long-term (22 to 36 weeks) E2 administration rats (Fig. 1). Such long-term E2 administra-

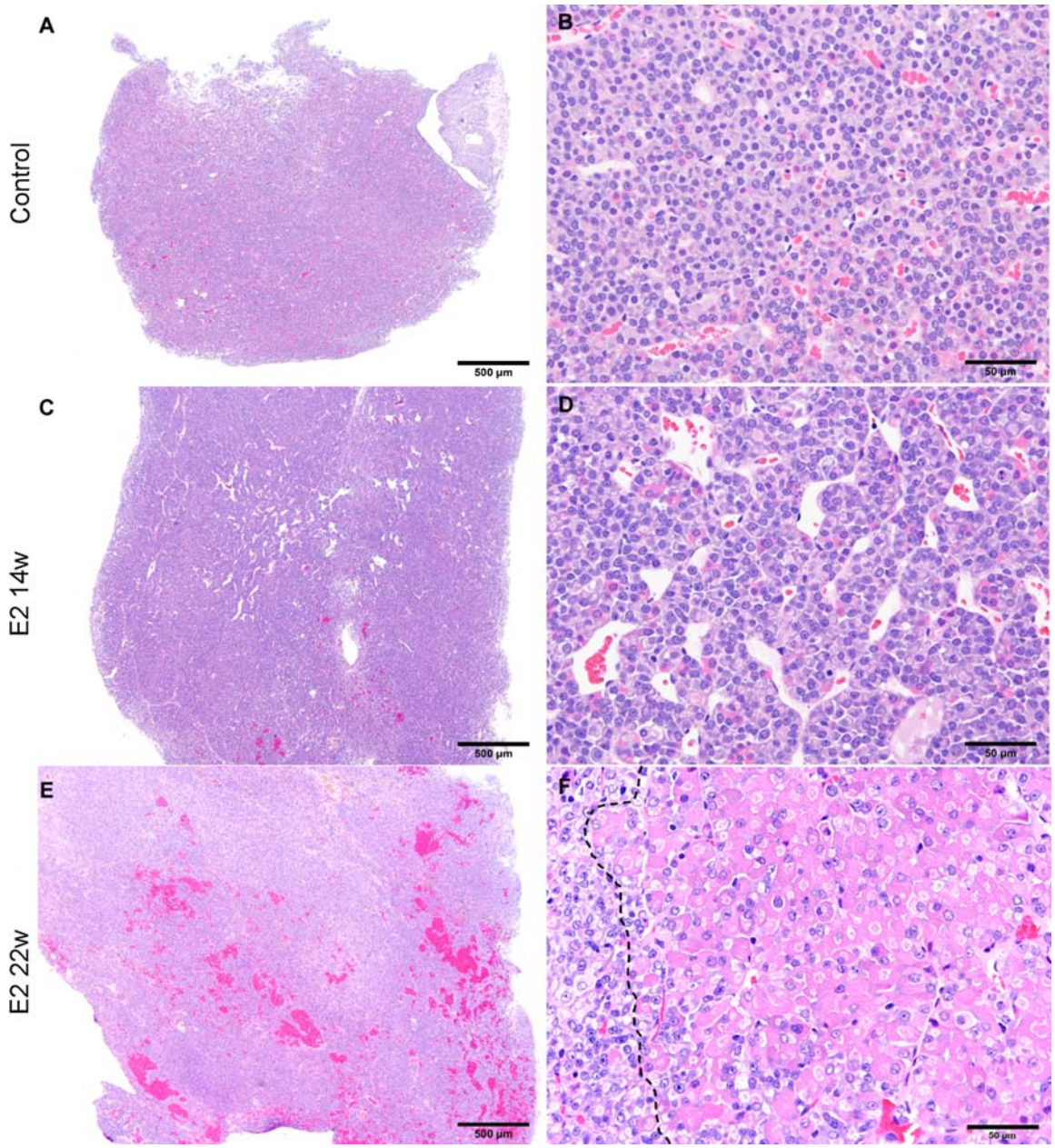

Fig. 2. Histopathological findings for pituitary multi-hormonal nodules in the rat. Sections were stained with H&E. **A, B**: Control group animals; **C, D**: E2 administration group (14-week administration); **E, F**: E2 long-term administration group (22-week administration). Vascular dilatation was observed in the E2 administration group and markedly in the long-term E2 administration group. In the long-term E2 administration group, neoplastic nodules were formed, in which there were large eosinophilic cells (right side of the dashed line).

tion was therefore demonstrated to promote the development of pituitary adenomas far larger than conventional prolactin-producing adenomas.

Macroscopic examination confirmed that all of the 11 animals that were injected with E2 for a long period incurred PRLomas that were subjected to histopathological examination after H&E staining following the conventional method. The histopathological examination disclosed that in the pituitaries of the long-term E2-administered rats blood vessels in a number of regions were remarkably dilated (Fig. 2). Additionally, as indicated by the dashed line, 10 of the 11 PRLomas from rats treated with E2 for a long period contained neoplastic nodules composed of large eosinophilic cells in a localized and multifocal fashion.

Immunohistochemical analysis of pituitary hormone expressions in multi-hormone producing neoplastic nodules

To explore pituitary hormone expressions, immunohistochemical staining was performed using anti-PRL anti-

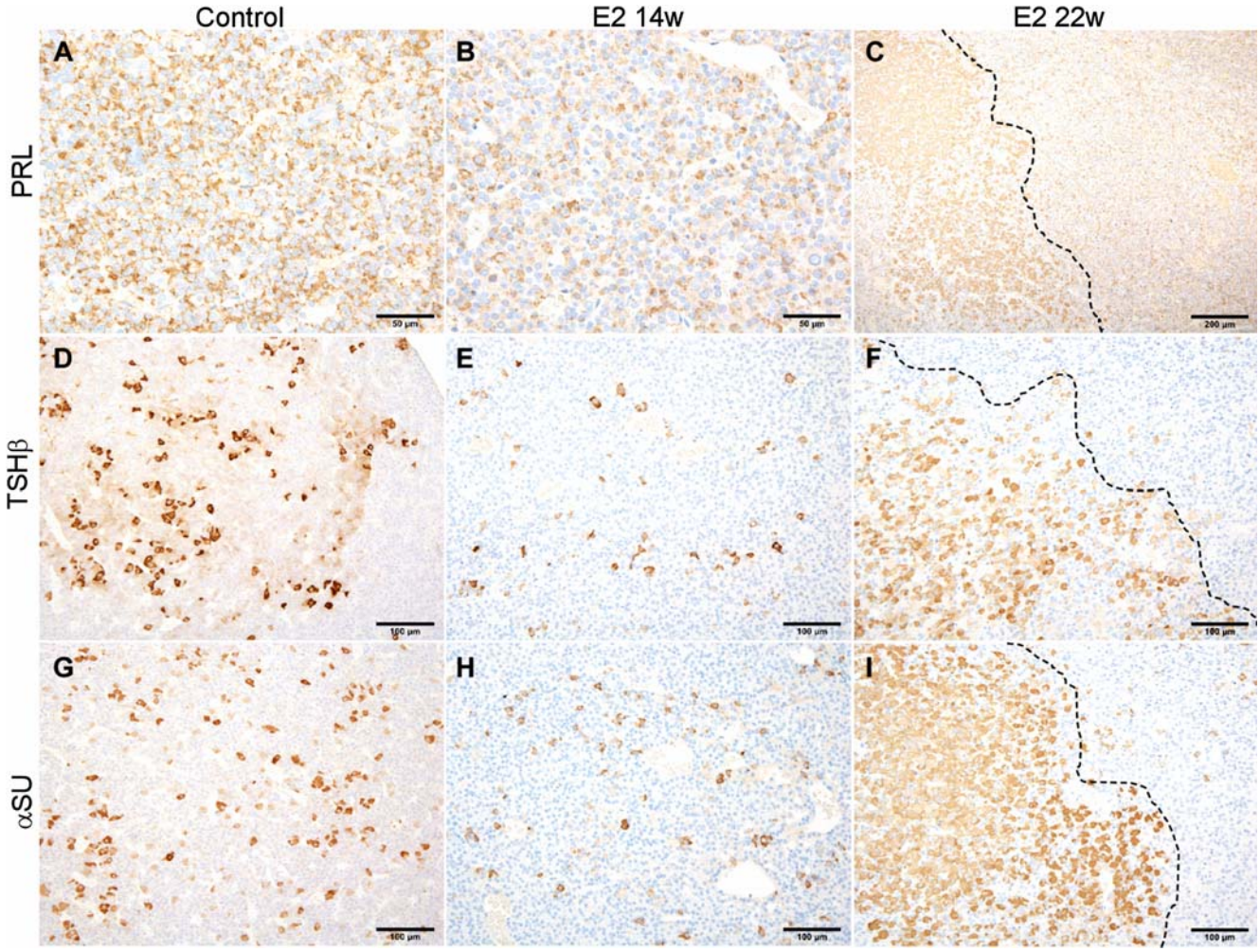

Fig. 3. Immunohistochemical analysis of pituitary hormones. Sections were stained with PRL (**A, B, C**), TSHβ (**D, E, F**), and αSU (**G, H, I**). In the control group, PRL-positive cells were localized in the whole pituitary. In the 14-week E2 group (prolactin-producing adenoma), PRL-positive cells were slightly decreased in number. In the long-term E2 administration group (22 to 36 weeks), almost all cells were positive for PRL. In the control group, TSHβ-positive cells were localized in the whole pituitary. In the 14-week E2 administration group, the number of TSHβ-positive cells was not more than 5%. In the long-term E2 administration group, almost all cells were positive for TSHβ in neoplastic nodules composed of large eosinophilic cells. In the control group, αSU-positive cells were localized in the whole pituitary. In the 14-week E2 group, the number of αSU-positive cells was not more than 5%. In the long-term E2 administration group, almost all cells were positive for αSU in multi-hormone producing neoplastic nodules.

body, anti-GH antibody, anti-TSHβ antibody, anti-αSU antibody, anti-LHβ antibody, anti-ACTH antibody, and anti-S-100 antibody. As shown in Figure 3, it was verified that the large eosinophilic cells in the neoplastic nodules surrounded by the dashed line were positive for PRL, TSHβ, and αSU. Results of simple staining are shown in Table 1. GH, LHβ, ACTH, and S-100 were negative (data not shown). No difference was observed in the immunoreactivity of PCNA and Ki67 between multi-hormone producing neoplastic nodules and non-nodular region (data not shown).

Since the large eosinophilic cells were immunohistochemically proven to be positive for PRL, TSHβ, and αSU, it was suggested that these factors might be co-expressed. Double immunostaining was therefore performed using combinations of various antibodies (Fig. 4). As a result, such large cells were found to coexpress PRL and TSHβ, PRL and αSU, and TSHβ and αSU. Results of the double immunostaining are shown in Table 2.

Analysis of gene expression with the use of laser-capture microdissection

Neoplastic nodules composed of large eosinophilic cells, non-nodule, and normal pituitary tissues were excised from rat PRLomas by the laser-capture microdissection method. Total RNAs from these nodules or tissues were analyzed for mRNA expression of PRL, GH, TSHβ and αSU, revealing that mRNA expression of TSHβ and αSU in proliferative lesions composed of large eosinophilic cells (multi-hormone producing sites) was significantly

Table 1. *Summary of immunohistochemistry analysis of hormones*

Experimental Group	Animal number	Administration weeks	stain-positive eosinophilic cell in proliferative nodular lesion						
			PRL	GH	TSHβ	αSU	LHβ	ACTH	S-100
E2 22-36w	1	22	+	–	+	+	–	–	–
	2	29	+	–	+	+	–	–	–
	3	36	+	–	+	+	–	–	–
	4	25	+	–	+	+	–	–	–
	5	25	No lesion	No lesion	No lesion	No lesion	No lesion	No lesion	No lesion
	6	25	+	–	+	+	–	–	–
	7	26	+	–	+	+	–	–	–
	8	26	+	–	+	+	–	–	–
	9	26	+	–	+	+	–	–	–
	10	26	+	–	+	+	–	–	–
	11	26	+	–	+	+	–	–	–

Table 2. *Summary of double immunohistochemistry analysis of hormones*

Experimental Group	Animal number	stain-positive eosinophilic cell in proliferative nodular lesion					
		TSHβ(+)PRL(+)	αSU(+)PRL(+)	TSHβ(+)αSU(+)	GH(+)PRL(+)	TSHβ(+)GH(+)	αSU(+)GH(+)
E2 22-36w	1	+	+	+	–	–	–
	2	+	+	+	–	–	–
	3	+	+	+	–	–	–
	4	+	+	+	–	–	–
	5	No lesion	No lesion	No lesion	No lesion	No lesion	No lesion
	6	+	+	+	–	–	–
	7	+	+	+	–	–	–
	8	+	+	+	–	–	–
	9	+	+	+	–	–	–
	10	+	+	+	–	–	–
	11	+	+	+	–	–	–

increased in comparison with the surrounding non-nodular region (Fig. 5). Notably, TSHβ and αSU were only minimally expressed in surrounding tumorous tissues but remarkably expressed only in multi-hormone producing neoplastic nodules.

IV. Discussion

Hitherto reported mainstream experimental animal models of multi-hormone producing pituitary tumors were genetically engineered animals such as knockout mice with genetic mutations of MEN1 or Prkarlα and/or transgenic mice with transcription factors such as PROP1 and PTTG [3, 4, 9, 34]. In this study, we clarified for the first time that administration of a hormone preparation induced multi-hormone producing neoplastic nodules in the pituitary.

The double immunostaining method disclosed that these neoplastic nodules contained many cells that simultaneously produced multiple hormones in pairs as exemplified by the pairing of "PRL and TSH" or of "PRL and αSU." The neoplastic nodules were selectively microdissected with the laser capture microdissection method to be compared regarding mRNA expression for hormones with other portions of the pituitary adenoma. It was then verified that mRNAs for PRL, TSH, and αSU were strongly expressed in the neoplastic nodules consistent with the results of immunostaining, revealing that multi-hormone production was induced at the transcription level. Interestingly, expression of mRNA for GH tended to increase, although not significantly, in the neoplastic nodules despite the fact that it was scarcely detected by immunostaining.

As mentioned above, such genetically engineered animals as Prkarlα knockout and PROP1 transgenic mice indeed develop multi-hormone producing pituitary adenomas but those adenomas generate hormones of GH, PRL, and TSH, which are assumed to be derived from the same cell lineage in terms of functional differentiation (GH-PRL-TSH production series). In contrast, the estrogen-induced pituitary adenoma in the current study characteristically produced hormones from different cell lineages like PRL and αSU, especially from the very same cells.

In the case of human pituitary adenoma as well, some cases reportedly expressed hormones overriding the cell lineages. This phenomenon is considered to be caused by

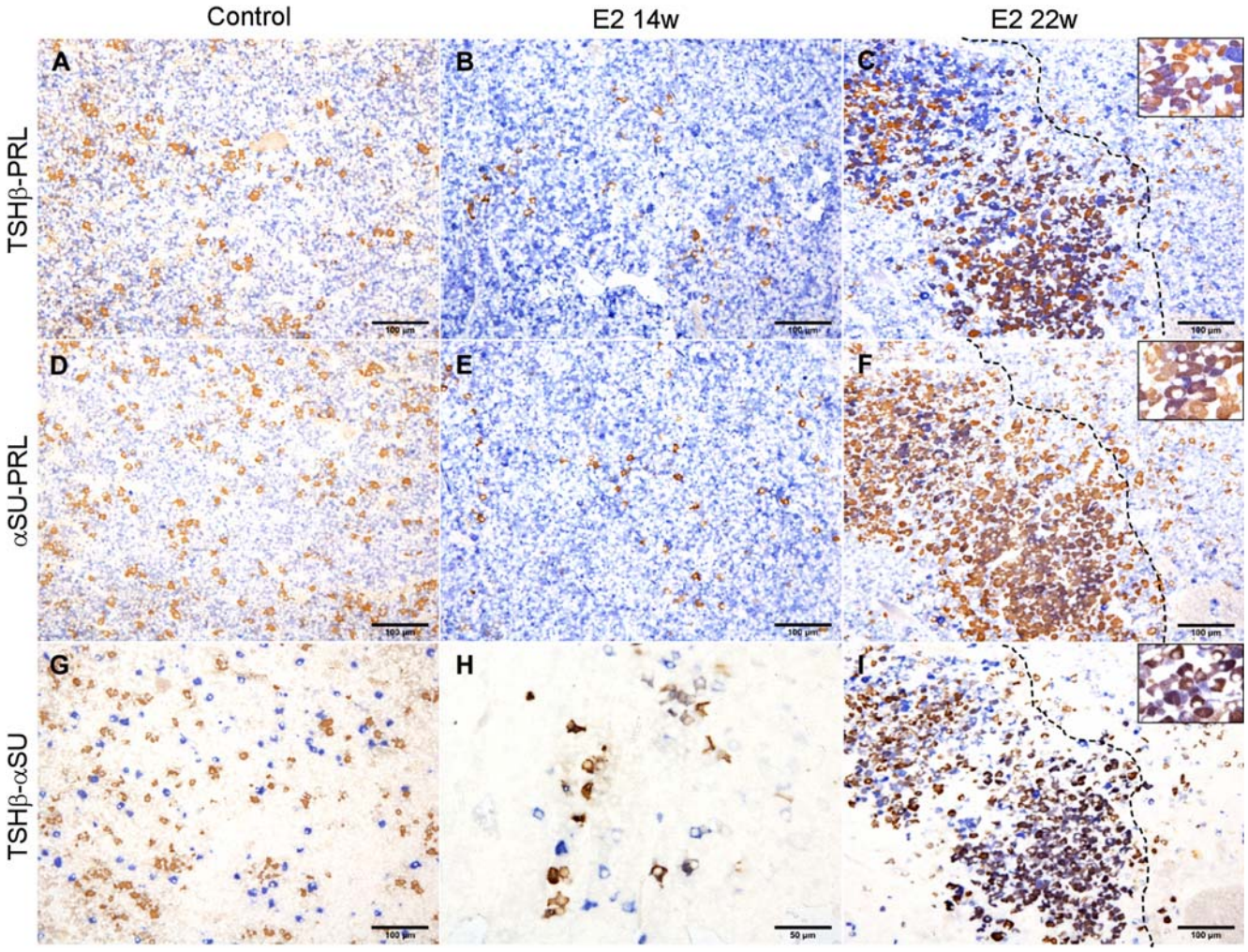

Fig. 4. Double immunohistochemistry of pituitary hormones. Sections were stained with TSHβ and PRL (**A, B, C**), αSU, and PRL (**D, E, F**), and TSHβ and αSU (**G, H, I**). TSHβ- or αSU-positive cells are stained brown, and PRL-positive cells are blue (**A, B, C, D, E, F**), and TSHβ-positive cells are stained brown, and αSU-positive cells are blue (**G, H, I**). In the control group, coexistence of PRL, TSHβ, and αSU was hardly recognized (**A, D, G**). In the 14-week E2 group, coexistence of TSHβ and αSU was partially observed (**B, E, H**). On the other hand, in the long-term E2 administration group (22 to 36 weeks), there were many cells in which TSHβ and PPL, αSU and PRL, and TSHβ and αSU coexisted (**C, F, I**).

trans-lineage differentiation due to abnormal expression of transcription factors. The expression of transcription factors was unclear in our case of multi-hormone producing neoplastic nodules. In the future we need to study details of the neoplastic nodules expression of transcription factors to control expression of Pit-1, ERα, and SF-1, regulators of pituitary hormone expression [7, 33, 35]. Such a future study is expected to clarify whether estrogen-induced multi-hormone producing adenomas are developed by the mechanism of trans-lineage differentiation due to abnormal expression of transcription factors similarly to human pituitary adenoma.

Estrogen has many effects on pituitary function, including regulation of most pituitary hormones, and proliferation of several pituitary cell types [10, 19, 28, 31]. Biological effects of estrogen are mediated by binding of hormone-bound ER dimers to specific estrogen-responsive regions in hormonal-regulated genes and initiating changes in gene transcription [16]. Clinically, elevated E2 levels during pregnancy are associated with symptomatic pituitary adenoma enlargement in up to 30% of women with macroadenomas [11]. In general, the highest levels of ERα mRNA and protein have been among PRLomas [5]. The mitogenic effects of estrogen in pituitary tumor have long been known to be mediated through ERα. Estrogen can also induce tumors in a variety of animal models, among which prolactin-producing pituitary tumors can be induced in rats by long-term estrogen treatment [18, 23, 25]. Shukuwa *et al.* have reported that diethylstilbestrol (DES), an estrogen analogue acting as an agonist of estrogen, induces proliferation of PRL cells and transdifferentiation of FSH/LH cells to PRL cells [27]. They also found that the induction of ERβ by DES is through the function of ERα. It is known that the expression of ERα markedly decreased by long-

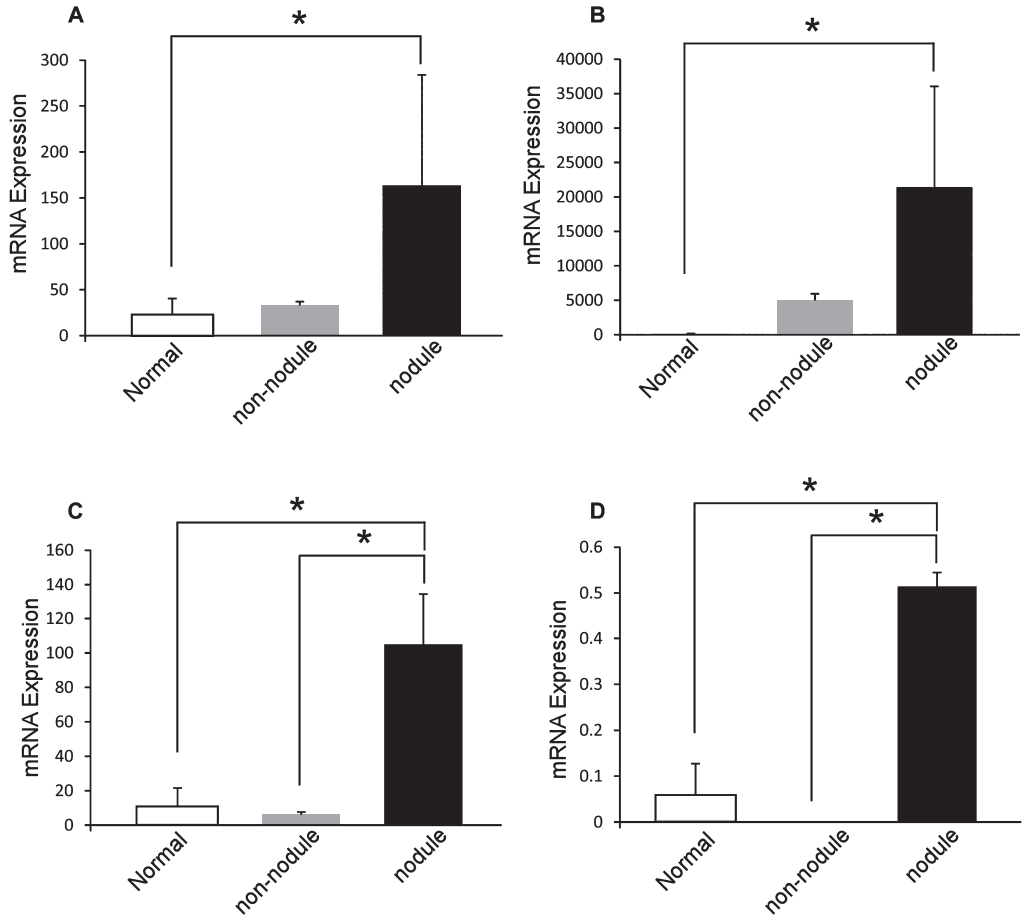

Fig. 5. Laser capture microdissection and quantitative real-time RT-PCR analysis. (**A**) GH, (**B**) PRL, (**C**) αSU, and (**D**) TSHβ were analyzed by quantitative real-time RT-PCR. Analysis of mRNA expression of GH, PRL, αSU, and TSHβ was performed in normal tissues, non-nodules, and multi-hormone producing nodules, respectively. Expressions of mRNA for GH, PRL, αSU, and TSHβ were significantly increased in multi-hormone producing nodules comparison with normal tissues and non-nodules. *P<0.05

term treatment of estrogen. Our preliminary experiments showed that ERα is rarely expressed both multi-hormone producing nodule and non-multi-hormone producing nodule. The expression of ERβ in multi-hormone producing nodules, however, is still unknown. The role of ERβ in the pathogenesis of multi-hormone producing pituitary cells is of particular interest. Future studies should focus on the expression of ERα and ERβ isoforms because they may have differential action on the formation of multi-hormone producing neoplastic nodules.

Since it is known that estrogen induces p27 degradation depending on the MAP kinase cascade [12] and that p27 suppresses SOX2 expression [17], it is surmised that these growth-modulatory factors and transcription factors played a key role in the development of neoplastic nodules in the current study. In order to assess the growth activity, cells were assessed for positivity for Ki67 and PCNA by immunohistochemistry. However, intensive growth activity was not seen in multi-hormone producing neoplastic nodules compared with non-nodular region. Based on this result, it appeared that there was no correlation between increased growth and pituitary hormone producing capabilities. To create an estrogen-induced rat prolactinoma, highly estrogen-sensitive Fischer rats are often used [2]. We, however, think that SD rats could develop multi-hormone producing neoplastic nodules because of their ability to tolerate long-term administration of estrogen. Future studies are required to decide whether multi-hormone producing neoplastic nodules are developed specifically in SD rats or whether these neoplastic nodules are induced by long-term estrogen administration irrespective of rat species.

It was clarified that long-term estrogen administration induced neoplastic nodules composed of large eosinophilic cells. These neoplastic nodules expressed hormones of PRL, TSHβ, and αSU, simulating human multi-hormone producing pituitary adenoma. In addition, the large eosinophilic cells, components of the neoplastic nodules, uniquely expressed multiple hormones in the same cell and at the same time. We expect the current animal model to be useful for pathogenesis analysis and therapeutic agent development concerning human multi-hormone producing pituitary adenoma.

V. Acknowledgments

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