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Morphologic and Molecular Analysis of Estrogen-Induced Pituitary Tumorigenesis in Targeted Disruption of Transforming Growth Factor-β Receptor Type II and/or p27 Mice

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The critical genes and products involved in estrogen-induced tumorigenesis of the pituitary gland were investigated in heterozygous transforming growth factor-β (TGF-β) receptor type II and p27 knockout mouse models. *Tgfbr2*(+/–), p27(+/–);*Tgfbr2*(+/–), and p27(+/–) mice and C57BL/6J wild-type mice received sc implantation of estrogen or placebo pellets for 16 or 25 wk, after which the mice were sacrificed and their pituitary glands removed for examination. The bromodeoxyuridine labeling indexes in tissue from both the anterior and intermediate pituitary lobes from p27 (+/–) and *Tgfbr2*(+/–);p27(+/–) mice were significantly higher than those from wild-type and *Tgfbr2*(+/–) mice after treatment with estrogen for 16 wk. Pituitary tumorigenesis was significantly accelerated in *Tgfbr2*(+/–), p27(+/–), and *Tgfbr2*(+/–);p27(+/–) mice compared with wild-type mice after treatment with estrogen for 16 wk. Pituitary tumorigenesis was not accelerated in *Tgfbr2*(+/–);p27(+/–) mice compared with *Tgfbr2*(+/–) or p27(+/–) mice. Expression of TGF-β receptor type II mRNA was lower in the pituitary gland of *Tgfbr2*(+/–) mice than in wild-type mice before estrogen treatment and was significantly reduced after treatment. Pituitary tumorigenesis is accelerated in mice with severe TGF-β resistance, and greatly accelerated in mice with TGF-β resistance combined with decreased p27 expression compared with wild-type mice. Both the TGF-β receptor type II gene and p27 gene and their products are involved in estrogen-induced tumorigenesis.

Key Words: Transforming growth factor-β RII gene; p27 gene; tumorigenesis; pituitary; knockout mice.

Introduction

Pituitary tumorigenesis may be caused by abnormalities in the expression of cell-cycle regulatory genes *(1)*. However, our previous investigations of the expression of cell-cycle regulatory genes in pituitary adenoma have rarely identified mutation of genes such as c-*myc (2)*, retinoblastoma gene *(3)*, p53 gene *(4)*, transforming growth factor-β (TGF-β) receptor type I (TGF-β RI) and type II (TGF-β RII) *(5)*, and p21 and p27 genes *(6)*. We also found that the expression of TGF-β RII protein was increased despite the absence of an active mutation of this gene *(1,5)*, and that expression of p27 was decreased compared to the normal pituitary tissue in most cases of pituitary adenoma *(1)*.

Expression of p27 protein in normal rat pituitary gland significantly decreases after treatment with 10^{-9} *M* TGF-β1 *(7)*, suggesting that decreased expression of p27 protein in human pituitary adenomas may result from increased expression of TGF-β RII, which inhibits expression of p27. However, the mechanism of increased expression of TGF-β RII in human pituitary adenomas and whether it is a primary or secondary change remains unclear. TGF-β1 suppresses the early stages of tumor formation in vivo for most cancers, but the tumor cells appear to escape from TGF-β-dependent growth inhibition at some point during tumor progression *(8,9)*. Paradoxically, many transformed cells or advanced invasive tumor cells constitutively produce TGF-β. Resistance to TGF-β inhibition is a result of inactivation of the TGF-β pathway, which may be owing to homozygous gene loss, gross gene rearrangement, truncated transcription of the TGF-β RII gene (*Tgfbr2*) *(9–12)*, or rearrangement of TGF-β RI and loss of mRNA *(9–13)*. Loss of TGF-β RII mRNA expression occurs in small-cell lung cancer *(14)*, esophageal cancer *(15)*, and thyroid cancer *(16)* despite the absence of structural alterations within the *Tgfbr2*. As TGF-β has two different and opposite functions in the process of tumor progression, the study of TGF-β-resistant cells may help to elucidate the mechanisms that leads to increased expression of TGF-β RII in human pituitary adenomas.

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The heterozygous *Tgfbr2* knockout (*Tgfbr2*[+/–]) mouse provides the ideal animal model for TGF-β resistance *(17)*. The homozygous *Tgfbr2* knockout (*Tgfbr2*[–/–]) mouse dies at about 10.5 d of gestation owing to defects in the yolk sac hematopoiesis and vasculogenesis, but the *Tgfbr2*(+/–) mouse is developmentally normal. TGF-β-1 and TGF-β RII protein and mRNA levels decrease in the pituitary gland during estrogen (estradiol 17β; E_2)-induced tumorigenesis *(18)*, indicating that treatment with E_2 induces TGF-β resistance.

Spontaneous development of pituitary tumors often occurs in the homozygous p27 knockout (p27[-/-]) mouse *(19–21)*, which indicates that p27 deletion can cause neoplastic growth of pituitary cells. Previously, we found that expression of p27 was undetectable in most human pituitary adenomas *(1)* although no p27 gene mutation was found *(6)*. TGF-β stimulation also causes the downregulation of p27 expression *(22)*; therefore, pituitary tumorigenesis in the heterozygous p27 knockout (p27[+/–]) mouse, in which p27 production is reduced, under conditions of TGF-β resistance is of interest.

The present study investigated the effect of severe TGF-β-resistant conditions induced by administration of estrogen on the incidence of pituitary tumorigenesis in the *Tgfbr2* (+/–) mouse, the p27(+/–) mouse, and the *Tgfbr2*(+/–);p27(+/–) mouse to examine the interaction between the p27 gene and *Tgfbr2* and gene products during E_2-induced pituitary tumorigenesis. The experimental groups were defined according to the genotype and the period of E_2 treatment as follows: group I, wild-type mice without E_2 treatment ($n = 5$); group II, wild-type mice after 16 wk of E_2 treatment ($n = 8$); group III, wild-type mice after 25 wk of E_2 treatment ($n = 7$); group IV, *Tgfbr2*(+/–) mice without E_2 treatment ($n = 5$); group V, *Tgfbr2*(+/–) mice after 16 wk of E_2 treatment ($n = 6$); group VI, *Tgfbr2*(+/–) mice after 25 wk of E_2 treatment ($n = 7$); group VII, p27(+/–) mice without E_2 treatment ($n = 3$); group VIII, p27(+/–) mice after 16 wk of E_2 treatment ($n = 7$); group IX, *Tgfbr2*(+/–); 27(+/–) mice after 16 wk of E_2 treatment ($n = 10$). Histopathologic examination and molecular analysis were used to evaluate the responses of the pituitary tissues.

Results

Macroscopic and Histologic Findings

The mean weight of the pituitary gland of p27(+/–) mice without E_2 treatment was significantly greater ($p < 0.01$) compared with those of wild-type and *Tgfbr2*(+/–) mice without E_2 treatment (Fig. 1A). The mean weights of the pituitary gland of p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice after E_2 treatment for 16 wk were significantly greater compared with those of wild-type and *Tgfbr2*(+/–) mice. The mean weight of the pituitary gland of *Tgfbr2*(+/–) mice after E_2 treatment for 25 wk was significantly greater compared with that of wild-type. The mean weights of the pituitary glands of mice of all genotypes after E_2 treatment for 16 or 25 wk were significantly greater compared with that of wild-type mice without E_2 treatment. The mean weight of the pituitary gland showed no significant difference between wild-type and *Tgfbr2*(+/–) mice after E_2 treatment for 16 wk.

Morphologic Analysis

The mean cell area in the anterior lobe of the pituitary gland of mice without E_2 treatment showed no significant differences between genotypes (Fig. 1B). The mean cell area in the anterior lobe of *Tgfbr2*(+/–) mice after E_2 treatment for 16 wk was significantly larger than that of *Tgfbr2* (+/–);p27(+/–) mice after the same treatment. The mean cell areas in the anterior lobe of mice after both durations of E_2 treatment were significantly larger than those of mice of all genotypes without E_2 treatment.

The mean cell area in the intermediate lobe of the pituitary gland of *Tgfbr2*(+/–) mice without E_2 treatment was significantly larger than that of p27(+/–) mice without E_2 treatment (Fig. 1C). The mean cell area in the intermediate lobe showed no significant difference between mice of different genotypes after E_2 treatment for 16 or 25 wk. The mean cell areas in the intermediate lobe of mice after E_2 treatment were significantly larger than those of mice without E_2 treatment for all genotypes and both durations of E_2 treatment (Fig. 2).

The bromodeoxyuridine (BrdUrd) labeling index in the anterior lobe of the pituitary gland of mice without E_2 treatment showed no significant difference between genotypes (Fig. 1D). The BrdUrd labeling indexes in the anterior lobe of p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice after E_2 treatment for 16 wk were significantly higher than those of wild-type and *Tgfbr2*(+/–) mice. The BrdUrd labeling index in the anterior lobe of *Tgfbr2*(+/–) mice after E_2 treatment for 25 wk was significantly higher than that of wild-type mice. The BrdUrd labeling index in the anterior lobe showed no significant difference between wild-type mice without E_2 treatment and after E_2 treatment for both durations. The BrdUrd labeling index in the anterior lobe of *Tgfbr2*(+/–) mice after E_2 treatment for 25 wk was significantly higher than those of *Tgfbr2*(+/–) mice without E_2 treatment and after E_2 treatment for 16 wk. The BrdUrd labeling index in the anterior lobe of p27(+/–) mice after E_2 treatment for 16 wk was significantly higher than that of p27(+/–) mice without E_2 treatment.

The BrdUrd labeling index in the intermediate lobe of the pituitary gland of mice without E_2 treatment showed no significant difference between genotypes (Fig. 1E). The BrdUrd labeling indexes in the intermediate lobe of p27 (+/–) and *Tgfbr2*(+/–);p27(+/–) mice after E_2 treatment for 16 wk were significantly higher than those of wild-type and *Tgfbr2*(+/–) mice. The BrdUrd labeling indexes in the intermediate lobe of wild-type mice after E_2 treatment for 16 and 25 wk were significantly higher than that of wild-type mice without E_2 treatment. The BrdUrd labeling index

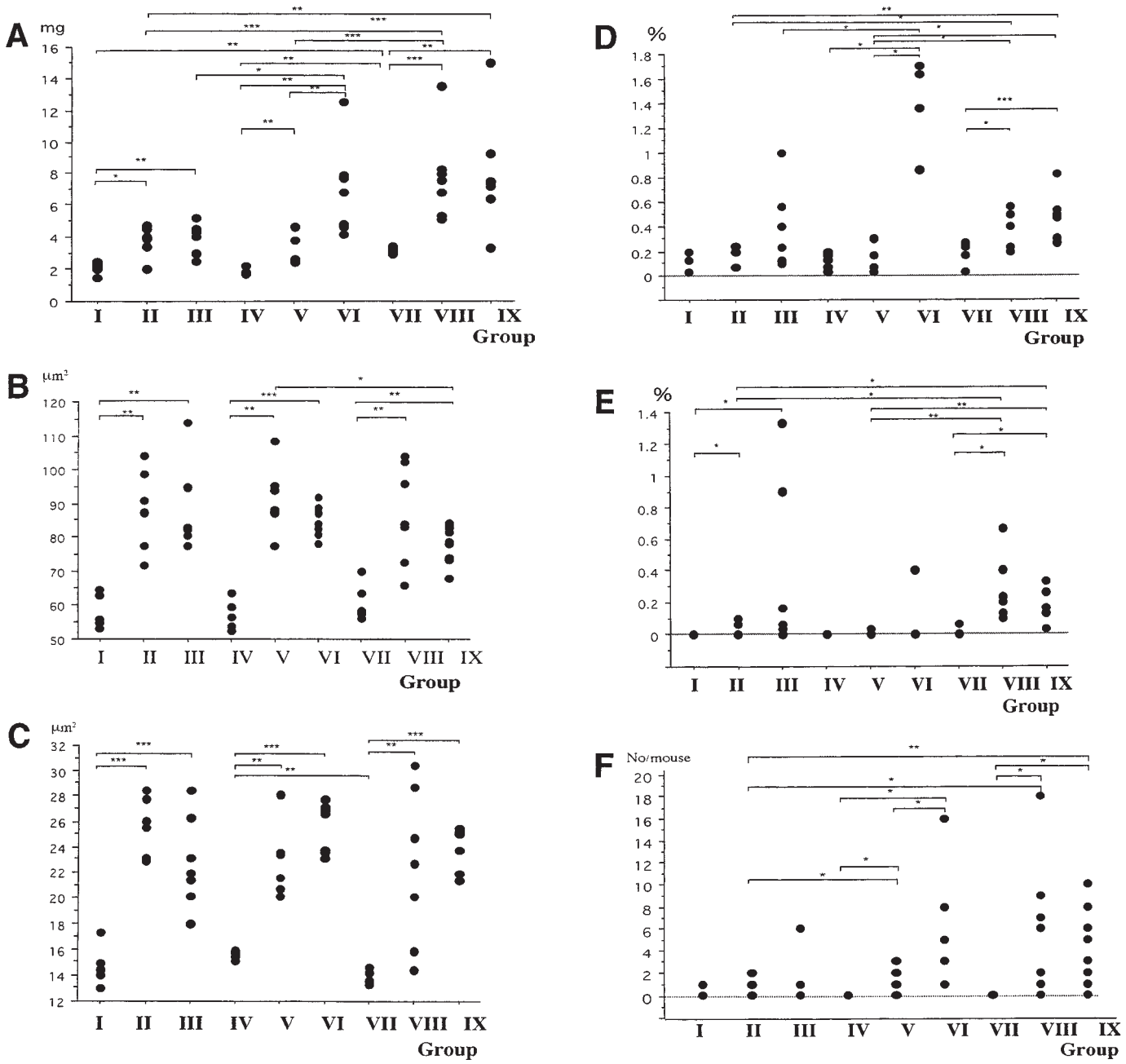

Fig. 1. Changes in pituitary weight and morphometric characteristics of pituitary gland after estrogen (E_2) treatment in mice of various genotypes. Group I, wild-type mice without E_2 treatment ($n = 5$); group II, wild-type mice after 16 wk of E_2 treatment ($n = 8$); group III, wild-type mice after 25 wk of E_2 treatment ($n = 7$); group IV, *Tgfbr2*(+/–) mice without E_2 treatment ($n = 5$); group V, *Tgfbr2*(+/–) mice after 16 wk of E_2 treatment ($n = 6$); group VI, *Tgfbr2*(+/–) mice after 25 wk of E_2 treatment ($n = 7$); group VII, p27(+/–) mice without E_2 treatment ($n = 3$); group VIII, p27(+/–) mice after 16 wk of E_2 treatment ($n = 7$); group IX, *Tgfbr2*(+/–);p27(+/–) mice after 16 wk of E_2 treatment ($n = 10$). Values are the mean ± SD. (**A**) Weight of pituitary gland; (**B**) mean cell area in anterior lobe of pituitary gland; (**C**) mean cell area in intermediate lobe of pituitary gland; (**D**) BrdUrd labeling index in anterior lobe of pituitary gland; (**E**) BrdUrd labeling index in intermediate lobe of pituitary gland; (**F**) adenoma formation in anterior lobe of pituitary gland. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

in the intermediate lobe showed no significant difference between wild-type mice after E_2 treatment for 16 and 25 wk. There was no significant difference in the BrdUrd labeling index in the intermediate lobe of *Tgfbr2*(+/–) mice for both durations of E_2 treatment. The BrdUrd labeling index in the intermediate lobe of p27(+/–) mice after E_2 treatment for 16 wk was significantly higher than that of p27(+/–) mice without E_2 treatment.

Spontaneous pituitary tumor (i.e., without E_2 treatment) was observed in the anterior lobe of the pituitary gland in

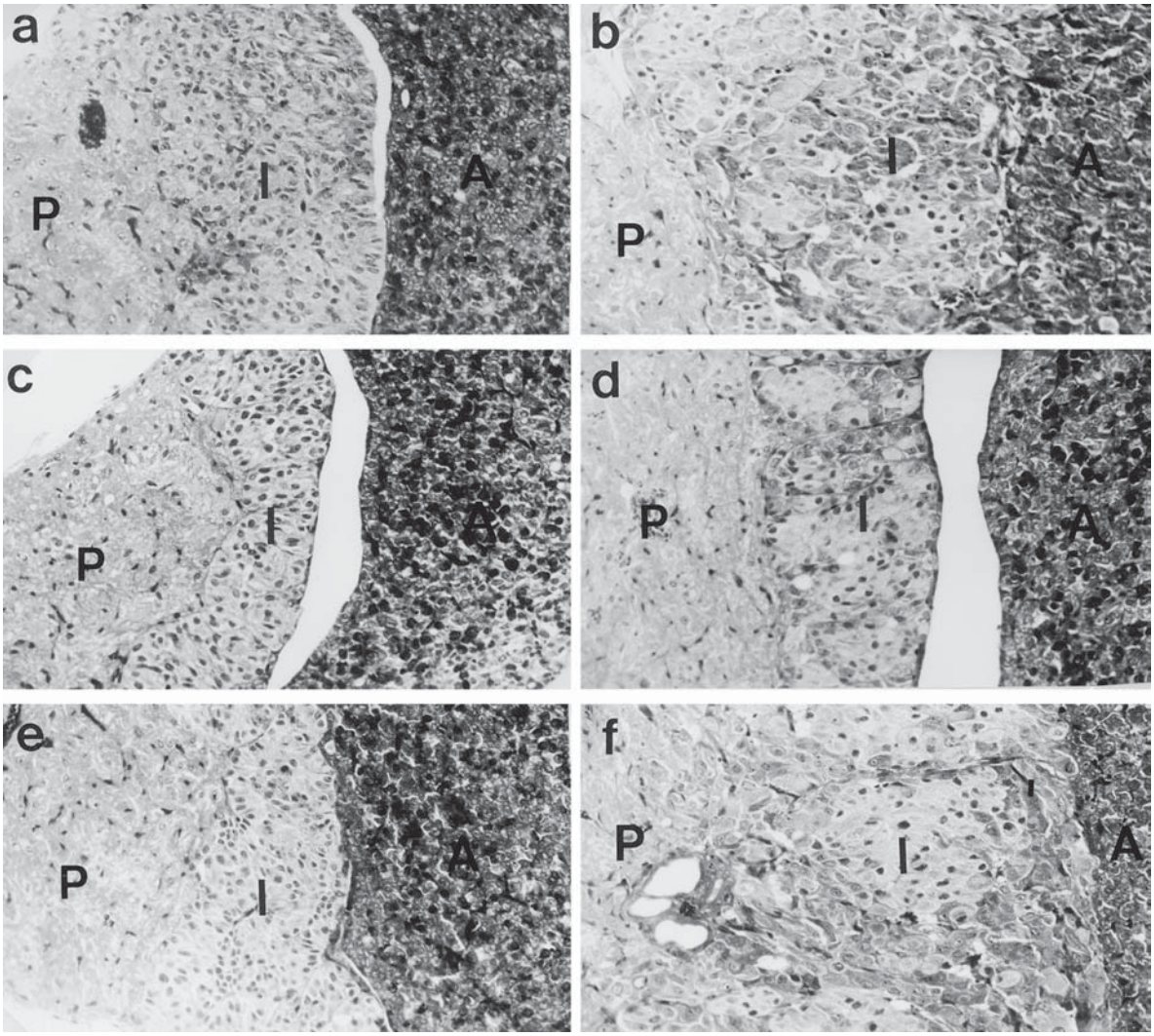

Fig. 2. Photomicrographs of pituitary gland of control mice and mice treated with estrogen for 16 wk. Estrogen treatment caused marked cellular hypertrophy in the intermediate lobes. (**A**) Group I; (**B**) group II; (**C**) group IV; (**D**) group V; (**E**) group VII; (**F**) group VIII. A, anterior lobe; I, intermediate lobe; P, posterior lobe.

only one mouse in group I, which was a prolactin (PRL)- and thyroid-stimulating hormone (TSH)-producing adenoma (Fig. 1F). There was no significant difference in pituitary tumorigenesis among groups I, IV, and VII. Pituitary tumor in the anterior lobe in mice after E_2 treatment for 16 wk was significantly higher in groups V, VIII, and IX than in group II. This indicates that pituitary tumorigenesis was significantly accelerated in *Tgfbr2*(+/–), p27(+/–), and *Tgfbr2*(+/–);p27 (+/–) mice compared with wild-type mice. There was no significant difference among groups V, VIII, and IX and no significant difference between mice after E_2 treatment for 25 wk (groups III and VI). Longer periods of E_2 treatment tended to cause more adenomas irrespective of genotype. There was a significant correlation ($p < 0.05$) between the period of E_2 treatment and the number of adenomas found in *Tgfbr2*(+/–) mice. The number of adenomas in mice after E_2 treatment for 16 wk (groups VIII and IX) was significantly larger than in mice without E_2 treatment (groups IV and VII). However, there was no significant difference among groups V, VIII, IX. Therefore, heterozygous loss of both *Tgfbr2* and p27 was not associated with accelerated formation of pituitary tumor compared with mice with heterozygous loss of either *Tgfbr2* or p27.

Pituitary tumor in the intermediate lobe of the pituitary gland was observed in one of seven wild-type mice after E_2 treatment for 25 wk, one of seven *Tgfbr2*(+/–) mice after E_2 treatment for 25 wk, and three of eight *Tgfbr2*(+/–); p27(+/–) mice after E_2 treatment for 16 wk. These five adenomas were immunopositive for adrenocorticotropic hormone (ACTH), β-endorphin, and α-melanocyte-stimulating hormone and negative for other pituitary hormones, which suggested an origin in the intermediate lobe. The incidence of intermediate lobe tumor tended to be higher in *Tgfbr2*(+/–); p27(+/–) mice than the other genotypes.

Table 1
Immunohistochemical Characteristics of Pituitary Tumors[a]

	Genotype	E_2 treatment (wk)	PRL	PRL/TSH	PRL/GH/TSH	GH/PRL	ACTH	Total
Group I	Wild type	—	0	1	0	0	0	1
Group II	Wild type	16	0	3	0	0	0	3
Group III	Wild type	25	10	2	1	0	1	14
Group IV	*Tgfbr2*(+/–)	—	0	0	0	0	0	0
Group V	*Tgfbr2*(+/–)	16	11	1	0	0	0	12
Group VI	*Tgfbr2*(+/–)	25	27	1	2	11	1	42
Group VII	p27(+/–)	—	0	0	0	0	0	0
Group VIII	p27(+/–)	16	32	0	1	10	0	43
Group IX	*Tgfbr2*(+/–); p27(+/–)	16	32	2	0	1	3	38
Total			112	10	4	22	5	153

[a]Numbers represent tumor multiplicity.

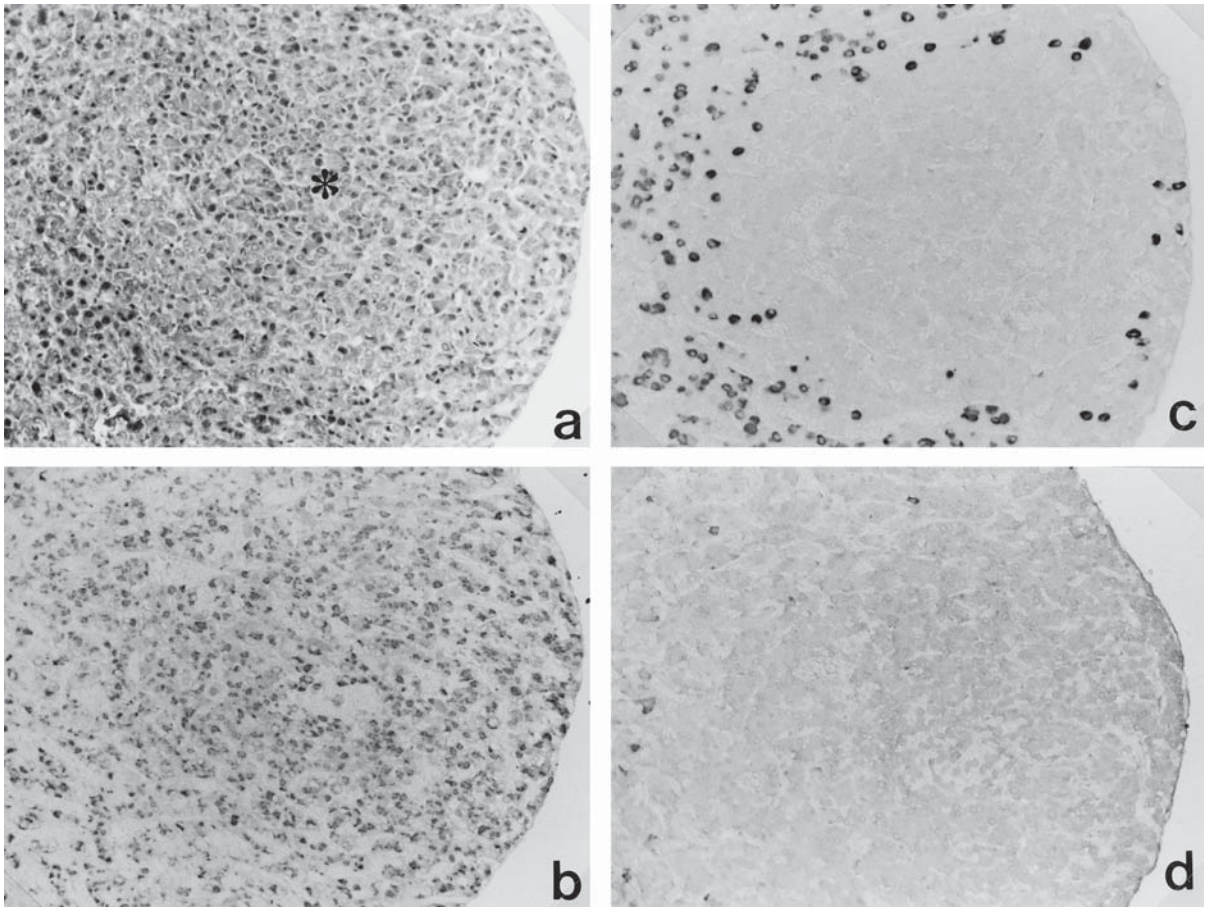

Fig. 3. Photomicrographs showing estrogen-induced pituitary adenoma in group II (original magnification ×200). (**A**) Hematoxylin and eosin staining, *pituitary adenoma; (**B**) immunohistochemical staining showing positive reaction for PRL; (**C**) immunohistochemical staining showing negative reaction for growth hormone (GH); (**D**) immunohistochemical staining showing negative reaction for TSH-β.

Immunohistochemical Staining

Table 1 presents the results of immunohistochemical staining. The incidence of pure PRL-producing tumor (Fig. 3) was higher in knockout mice than in wild-type mice.

Expression of mRNA

Expression of mRNA for the control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was almost the same in the wild-type and *Tgfbr2*(+/–) mice without E_2 treatment.

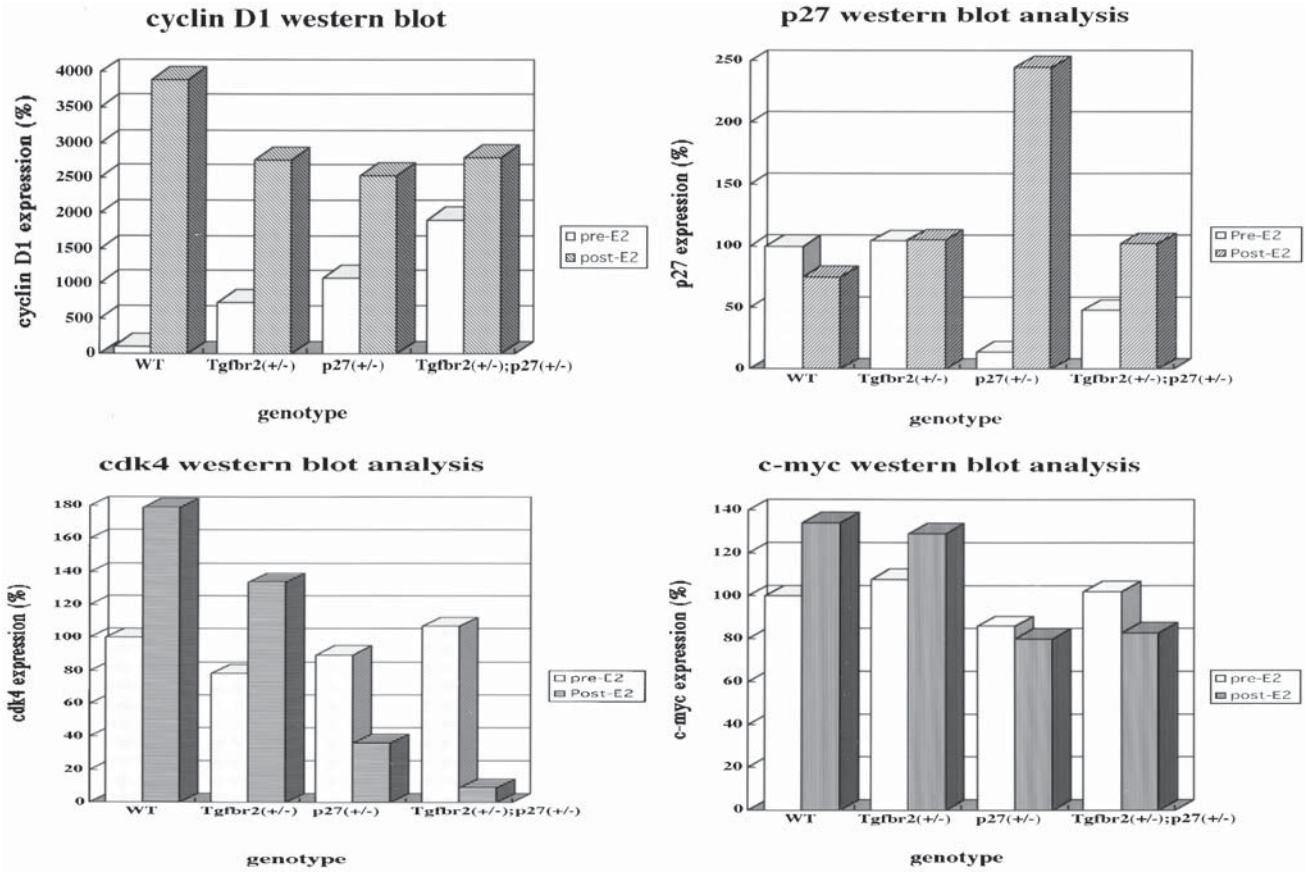

Fig. 4. Quantitative analysis of visualized protein bands (Western blotting) from pituitary gland before (**A,B**) and after estrogen treatment for 2 mo (**C,D**). WT, wild type.

Expression of mRNA for *Tgfbr2* was detectable in wild-type mice, but not in *Tgfbr2*(+/–) mice up to 28 cycles of polymerase chain reaction (PCR) amplification. Thus, there was a marked difference in the expression of *Tgfbr2* mRNA between the wild-type and *Tgfbr2*(+/–) mice. mRNA for G3PDH was detected in the wild-type and *Tgfbr2*(+/–) mice after E_2 treatment for 2 wk, but mRNA for *Tgfbr2* was not detected in either wild-type or *Tgfbr2*(+/–) mice after E_2 treatment for 2 wk. These results indicate that the level of mRNA for *Tgfbr2* in *Tgfbr2*(+/–) mice without E_2 treatment is lower than that in wild-type mice. The level of mRNA for *Tgfbr2* was greatly decreased after E_2 treatment compared with before E_2 treatment.

Expression of Nuclear Protein

Expression of cyclin D1 was increased in the pituitary glands of *Tgfbr2*(+/–), p27(+/–), and *Tgfbr2*(+/–);p27(+/–) mice before E_2 treatment compared with wild-type mice (Fig. 4A). Expression of cyclin D1 expression was greatly increased after E_2 treatment for 2 mo in all genotypes.

Expression of cdk4 in the pituitary glands of wild-type, *Tgfbr2*(+/–), p27(+/–), and *Tgfbr2*(+/–);p27(+/–) mice was almost the same before E_2 treatment (Fig. 4B). Expression of cdk4 in the pituitary glands of wild-type and *Tgfbr2*(+/–) mice increased after E_2 treatment for 2 mo but markedly decreased in p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice.

Expression of p27 protein in the pituitary glands of wild-type, *Tgfbr2*(+/–), and *Tgfbr2*(+/–);p27(+/–) mice did not show remarkable differences before and after E_2 treatment (Fig. 4C). Expression of p27 was only reduced in the pituitary gland of p27(+/–) mice before E_2 treatment and showed a remarkable increase after E_2 treatment for 2 mo.

Expression of c-*myc* in the pituitary glands of wild-type, *Tgfbr2*(+/–), p27(+/–), and *Tgfbr2*(+/–);p27(+/–) mice was almost the same before E_2 treatment (Fig. 4D). Expression of c-*myc* in the pituitary gland from wild-type and *Tgfbr2* (+/–) mice was increased after E_2 treatment for 2 mo but was unchanged in p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice. Therefore, the response of expression of cdk2 and c-*myc* to E_2 administration in wild-type and *Tgfbr2*(+/–) mice was quite different from that in p27(+/–) and *Tgfbr2*(+/–); p27(+/–) mice.

Discussion

Effect of Estrogen on Pituitary Cells

Measurement of pituitary weight cannot be used to evaluate the effect of E_2 on the pituitary gland since the effect

on the sinusoidal channels, which enlarge and increase in numbers, may form a significant part of the pituitary weight *(23,24)*. Therefore, morphometric analysis of pituitary cells is necessary to evaluate the effect of E_2 on pituitary cells.

The mean cell area in the anterior lobe of the pituitary gland increased but reached a maximum after E_2 treatment for 16 wk irrespective of genotype; thus, the degree of cellular hypertrophy in response to E_2 treatment was presumably the same. The BrdUrd labeling indexes in the anterior lobe of p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice after E_2 treatment for 16 wk were significantly higher than those of *Tgfbr2*(+/–) and wild-type mice. By contrast, the BrdUrd labeling index in the anterior lobe showed no significant difference between *Tgfbr2*(+/–) and wild-type mice after E_2 treatment for 16 wk, although the mean cell area in the anterior lobe increased significantly. Thus, the hyperplasia caused by E_2 treatment was mainly owing to cellular hypertrophy. Possibly, the incorporation of BrdUrd was not so increased after E_2 treatment because the cell cycle was not fast enough even after E_2 treatment for 16 wk to incorporate a significant amount of BrdUrd during exposure to BrdUrd for 1 h. Thus, p27 is more important than TGF-β RII for control of cell proliferation in the anterior lobe of the pituitary gland. However, E_2 treatment for as long as 25 wk resulted in a significantly higher BrdUrd labeling index in the anterior lobe of *Tgfbr2*(+/–) mice than that of wild-type mice. Thus, longer periods of E_2 treatment for *Tgfbr2*(+/–) mice resulted in a more significant increase in BrdUrd labeling index compared with wild-type mice.

The effect of E_2 treatment on the intermediate lobe of the pituitary gland is relatively well known in the hamster *(25–27)*. However, only one pituitary tumor arising from the intermediate lobe of the rat has been recorded in rats or mice *(28)*. The exact pathologic characteristics of intermediate lobe adenoma remain to be clarified. The mean cell area in the intermediate lobe increased and reached a maximum after E_2 treatment for 16 wk irrespective of genotype; thus, the degree of cellular hypertrophy in response to E_2 treatment was presumably the same. Our study demonstrated that the mean cell areas of both anterior and intermediate lobes showed significant increases after E_2 treatment. Five pituitary adenomas arising from the intermediate lobe were discovered. The *Tgfbr2*(+/–);p27(+/–) mouse tended to develop adenomas in the intermediate lobe more frequently than the other genotypes. The mean cell area and BrdUrd labeling index in the intermediate lobe showed no significant correlation with the period of E_2 treatment. The BrdUrd labeling indexes in the intermediate lobe of p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice after E_2 treatment for 16 wk were significantly higher than those of *Tgfbr2*(+/–) and wild-type mice. These results indicate that p27 is important in the regulation of cell proliferation in the intermediate lobe of the pituitary gland.

Susceptibility of Different Mouse Genotypes to Pituitary Tumorigenesis Induced by Administration of Estrogen

Preliminary experiments showed that pituitary tumorigenesis is greatly accelerated in *Tgfbr2*(+/–) mice by E_2 treatment *(29)*. The formation of microscopic adenomas in *Tgfbr2*(+/–) or p27(+/–) mice was significantly greater than in wild-type mice after E_2 treatment for 16 wk. Heterozygous loss of the DNA for the TGF-β RII or p27 genes obviously accelerated E_2-induced tumorigenesis in these knockout mice. However, the *Tgfbr2*(+/–);p27(+/–) mice did not show accelerated pituitary tumorigenesis compared with *Tgfbr2*(+/–) or p27(+/–) mice after E_2 treatment for 16 wk. Thus, the loss of *Tgfbr2*(+/–) and p27(+/–) genes resulted in no synergy in the acceleration of pituitary tumorigenesis. There was no significant difference in the formation of adenomas between *Tgfbr2*(+/–) and p27(+/–) mice after E_2 treatment for 16 wk; therefore, whether the *Tgfbr2* or p27 gene is more important in pituitary tumorigenesis remains unclear.

Cell susceptibility to neoplastic transformation may be increased by factors that modulate cell-cycle progression, including overexpression of cyclin D1 *(30–32)*, and TGF-β resistance, e.g., owing to inactivation of the TGF-β RII tumor suppressor gene *(33)*. The effects of E_2 treatment on cell-cycle regulatory factors are reported as follows: increased cyclin D1 expression accompanied by activation of cdk4 *(34,35)*, cdk-independent activation of estrogen receptor (ER) by cyclin D1 *(30)*, increased c-*myc* expression *(34)*, p27 expression induced in rat uterus *(36)*, but p27 expression unaltered in breast cancer cells *(34,35)*, and decreased TGF-β RI and RII mRNA and protein expression *(18,37)*. Therefore, E_2 induces both increased expression of cyclin D1 and decreased expression of TGF-β RI and RII, thus allowing escape from TGF-β-mediated growth control. These conditions eventually lead to the proliferation and neoplastic transformation of pituitary cells. In the present study, *Tgfbr2*(+/–) mice showed reduced expression of TGF-β RII compared with wild-type mice, and, thus, the pituitary tumorigenesis was strongly accelerated.

E_2 stimulation may have different effects on the expression of cdk4, p27, and c-*myc* in wild-type and p27-deficient mice. E_2 stimulation caused the opposite effect on the expression of cdk4, p27, and c-*myc* in mice with reduced p27 levels (p27[+/–] and *Tgfbr2*[+/–];p27[+/–] mice) compared with the effect in wild-type (p27[+/+]) mice (Fig. 4B–D). p27 is haplo-insufficiency for tumor suppression *(38)*, and reduced p27 levels could sensitize the pituitary cells to tumorigenic factors such as radiation and chemical carcinogens *(39)*. In particular, there was a marked increase in expression of p27 in the p27-deficient mice after E_2 treatment compared with wild-type mice. Reduced expression of c-*myc* may be followed by activation of p27 *(40)*. Expression of c-*myc* was induced by E_2 treatment in both wild-type and *Tgfbr2*(+/–) mice, but not in mice expressing low

levels of p27 (p27[+/–] and *Tgfbr2*[+/–];p27[+/–] mice) (Fig. 4D).Therefore, there may be an interaction between the c-*myc* and p27 genes, and, presumably, expression of p27 inversely increased in p27-deficient mice after E_2 treatment owing to the decreased expression of c-*myc (40)*.

Expression of cyclin D1 was remarkably high in *Tgfbr2* (+/–) and p27(+/–) mice before E_2 treatment compared with wild-type mice. Cyclin D1 activates ER *(30)* and hence may cause pituitary tumorigenesis in these mice, as well as reduce expression of TGF-β RII and thereby promote TGF-β resistance *(32)*. Expression of cyclin D1 after E_2 treatment had no correlation with the frequency of tumorigenesis (Figs. 1F and 4A), which indicates that other cell-cycle regulatory genes possibly interfere with the potential of cyclin D1 for tumorigenesis.

Expression of cdk4 was similar to that of c-*myc*, probably because cdk4 provides a direct link to the oncogenic effect of c-*myc (41)*. E_2-induced increase in expression of cyclin D1 is only associated with increased cdk4 activity in wild-type (p27[+/+]) mice *(31)*.

Recently, genetic evidence for the interactions between cyclin D1 and p27, and between cdk4 and p27, has been reported using double mutant cyclin D1–/–p27–/– and cdk4–/–p27–/– mice. Double mutant cyclin D1–/–p27–/– mice displayed reciprocal rescue of the phenotype in many but not all individuals, which indicates that the p27 and cyclin D1 functions are genetic and biochemical antagonists *(42)*. Similarly, double mutant cdk4–/–p27–/– fibroblasts displayed partial restoration of the kinetics of the G_0-S transition, indicating that cdk4 and p27 counteract each other genetically *(43)*. Therefore, the changes in the expression of p27, c-*myc*, and cdk4 after E_2 treatment compared with before in p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice are supposed to result from the interactions among p27, c-*myc*, cyclin D1, and cdk4 genes after E_2 treatment.

Pituitary gland of p27(+/–) and *Tgfbr2*(+/–);p27(+/–) mice showed significantly higher proliferative activity and higher incidence of pituitary tumorigenesis, suggesting that changes in cdk4 activation and c-*myc* induction by E_2 treatment might be involved in the different tumorigenicities in these knockout mice.

Materials and Methods

Preparation of Animal Models

Tgfbr2(+/–) *(17)* (a gift from Banyu Tsukuba Research Institute) and p27(+/–) male mice *(19)* (a gift from Nippon Roche Research Center) and 6-wk-old female C57BL/6J mice (wild-type) (a gift from Clea Japan Laboratory) were housed in a controlled environment (temperature, 23ºC; humidity, 50%; lighted 8:00 AM to 8:00 PM). The mice were given water containing 0.03 mg/mL of α-adrenergic antagonist (prazosin hydrochloride; Sigma, St. Louis, MO) to avoid urinary retention *(29)*. Male *Tgfbr2*(+/–) and p27 (+/–) mice were mated with female wild-type mice. *Tgfbr2* (+/–);p27(+/–) mice were obtained by crossing both heterozygous *Tgfbr2* and p27 knockout mice. Only female offspring were used after identification of their genotype. The study followed the guidelines for animal experimentation of Tohoku University School of Medicine.

Identification of Genotype

Genomic DNA was prepared from mouse tails after treatment with lysis buffer. Tissues were digested in a 500-mL solution containing 150 mg of proteinase K, 50 m*M* Tris-HCl (pH 8.0), 0.1 *M* NaCl, 20 m*M* Na_2EDTA, and 1% sodium dodecyl sulfate (SDS) overnight at 37ºC. The DNA was extracted with phenol/chloroform, then precipitated with cold ethanol overnight at –20ºC. The precipitate was separated by centrifugation. The pellets were dried and resuspended in TE buffer solution (10 m*M* Tris; 1 m*M* EDTA, pH 8.0) and stored at 4ºC until analysis.

The *Tgfbr2*(+/–) and *Tgfbr2*(+/+) genotypes were discriminated using allele-specific PCR with the following primer sets: bF (5'-TGATTATGGACTTGCTTCCCGG TA-3' from the intron 3 sequence) with bR (5'-CGAAGTC ACACAGGCAACAGGTCA-3' from the coding sequence of exon 4 corresponding to codons 391–398) to detect the wild allele and phosphoglycerate kinase (PGK) r (5'-CTAA AGCGCATGCTCCAGACT-3' from the PGK promoter sequence) with bF to detect the targeted allele (600 bp). The p27(+/–), p27(-/-), and p27(+/+) genotypes were discriminated using allele-specific PCR with the following primer sets: F-1 (5'-CCTGGAGCGGATGGACGCCAGA CA-3') with R-1 (5'-CACCAAATGCCGGTCCTCAGAG TT-3') to detect the wild allele (350 bp) and F-2 (5'-GGGC TTTAGAAATAGAGAATGCTG-3') with R-2 (5'-ATGC TCCAGACTGCCTTGGGAAAA-3') to detect the target allele (1100 bp).

PCR was performed using 100 ng of genomic DNA in 50 μL of PCR mixture, containing the following components: 20 m*M* dATP, 20 m*M* dGTP, 20 m*M* dTTP, 20 m*M* dCTP, 10 pmol of the corresponding primer, 2.0 m*M* $MgCl_2$, 20 m*M* Tris buffer (pH 8.4), 50 m*M* KCl, and 1.0 U of *Taq* DNA polymerase (Takara, Tokyo, Japan). PCR amplification (35 cycles) used a programmable temperature control system (PC-800; ASTEC, Fukuoka, Japan) under the following conditions: denaturation for 1 min, at 94ºC; annealing for 1 min, 15 s at 59–61ºC; and polymerization for 1 min, 30 s at 71ºC. However, the samples were denatured at 94ºC for 5 min in the first cycle, and the final polymerization step was performed for 5 min at 71ºC. The temperature for annealing was suitably adjusted for each segment. The PCR-amplified product solution (5 μL) was mixed with the same amount of loading solution (7 *M* uric acid, 50% sucrose, 10 m*M* Na_2EDTA, 0.1% bromophenol blue, 0.05% xylene alcohol) and then electrophoresed at 100 V for 25 min using Mupid-2 (Cosmo Bio, Tokyo, Japan) on 1.5% agarose gel containing ethidium bromide. The bands were visualized with an ultraviolet (UV) transilluminator and photographed.

The genotype of the *Tgfbr2* knockout mice was decided based on the presence of a band corresponding to the 600-bp amplified fragment indicating the *Tgfbr2*(+/–) genotype, or the absence of the band indicating the wild-type. The genotype of the p27 knockout mice was decided based on the presence of a band corresponding to the 350-bp amplified fragment indicating the wild-type, the presence of a band corresponding to the 1100-bp amplified fragment indicating the p27(-/-) genotype, and the presence of both 350- and 1100-bp bands indicating the p27(+/–) genotype.

Investigation of Spontaneous Pituitary Tumorigenesis

Estradiol pellets (1.5 mg, 90-d release type) (Innovative Research of America, Sarasota, FL) or placebo pellets (Innovative Research of America) were subcutaneously implanted under light phenobarbital anesthesia (50 mg/kg) through a small incision in the back. Additional pellets were implanted every 90 d after first implantation to maintain the plasma E_2 concentration at supranormal levels *(29)*.

After the required period of E_2 treatment, the mice were anesthetized with ether and injected intraperitoneally with 50 mg/kg of BrdUrd (Sigma). One hour after injection, they were sacrificed with ether anesthesia. The pituitary and adrenal glands were removed and weighed after fixation in 10% neutral buffered formalin at 4ºC for one night. Then the glands were embedded in paraffin for histologic examination.

Pituitary glands removed from seven *Tgfbr2*(+/–) and C57BL/6J mice without E_2 treatment and five *Tgfbr2*(+/–) and C57BL/6J mice after 2 wk of E_2 treatment were immediately frozen and used for measurement of mRNA expression. Pituitary glands removed from 10 *Tgfbr2*(+/–), C57BL/6J, p27(+/–), and *Tgfbr2*(+/–);p27(+/–) mice without E_2 treatment and after 2 mo of E_2 treatment were also immediately frozen and used for measurement of nuclear protein concentration.

Histologic Examination of Pituitary Glands

Morphologic Analysis

Microscopic adenoma was detected on serial stained sections as moderate disruption of parenchymal cells, cellular hypertrophy or clustering of anaplastic cells, loss of normal cell cord organization, presence of frequent mitotic figures, and cell clusters showing homogeneously positive or negative immunoreactivity for pituitary hormones.

Proliferation potential was measured as the percentage of BrdUrd-labeled cells (BrdUrd labeling index) by counting the number of positive cells among more than 1000 tumor cells in at least four representative high-power fields (×400) across the slide.

The area of the pituitary cells was measured on midaxial sections. The mean cell area in the anterior lobe was calculated as (0.03 mm^2)/(cell number per 0.03 mm^2). The mean cell area in the intermediate lobe was calculated as (0.04 mm^2)/(cell number per 0.04 mm^2).

The significance of differences in pituitary weight, number of adenomas, mean cell areas in the anterior and intermediate lobes, and BrdUrd labeling index among groups were tested by the nonparametric Mann-Whitney U test using the Statview-J 4.11 software package. A value of $p < 0.05$ was considered statistically significant.

Immunohistochemical Staining

Serial sections 3 μm thick were cut from a paraffin block. Every twentieth section was stained with hematoxylin and eosin. The rest of the sections were used for immunohistochemical staining by the avidin-biotin-peroxidase complex (ABC) method *(44)*. Sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Endogenous peroxidase activity was inhibited by treatment with 0.3% (v/v) hydrogen peroxidase in absolute methanol for 30 min. BrdUrd immunostaining used sections pretreated with 4 *N* HCl for 20 min to denature the DNA, followed by washing three times in boric acid and sodium borate buffer (pH 9.0 and 0.01 *M*) for 20 min to neutralize HCl *(45)*, and then treated with 0.05% protease (Sigma) in 0.01 *M* phosphate-buffered saline (PBS) (pH 7.6) for 1 min at 37ºC. All sections were rinsed in distilled water twice and in 0.01 mol/L of PBS (pH 7.2) for 5 min. After treatment for 30 min with normal goat or horse serum (1:20), the sections were incubated overnight at 4ºC with the primary antibodies as follows: polyclonal anti–human ACTH antibody (Dako, Glostrup, Denmark), polyclonal antirat PRL antibody (UCB, Poole, UK), polyclonal anti-GH antibody (Chemicon, Temecula, CA), polyclonal anti–mouse TSH antibody (UCB), polyclonal anti–rat luteinizing hormone antibody (UCB), polyclonal anti–mouse follicle-stimulating hormone antibody (UCB), polyclonal anti–human β-endorphin antibody (UCB), polyclonal anti–α-melanocyte-stimulating hormone antibody (UCB), and monoclonal anti–BrdUrd antibody (Becton Dickinson). The sections were then incubated with biotinylated antimouse or rabbit immunoglobulin G (IgG) at room temperature for 30 min, with the Vectastatin ABC kit (Vector, Burlingame, CA) at room temperature for 30 min, followed by incubation for 10 min in the presence of 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.05 mol/L of Tris buffer, pH 7.6. The sections were then counterstained with methylgreen for 30 min, dehydrated, cleared, and mounted.

The specificity of the polyclonal antibody was tested by immunoabsorption using the purified antigens to detect disappearance of the immunoreaction. Normal rabbit serum was substituted for the primary antibody and allowed to react as a negative control. Normal mouse pituitary tissue and small intestinal tissue with BrdUrd treatment were used as positive controls.

Measurement of mRNA Expression

mRNA was extracted from 100 mg of frozen pituitary gland using the QuickPrep Micro mRNA Purification Kit

(Pharmacia Biotech AB, Uppsala, Sweden). First-strand cDNA was also obtained using the Ready To Go T-Primed First Strand Kit (Pharmacia Biotech AB).

Quantitative PCR *(46,47)* was performed using the first-strand cDNA as a template to measure the expression of mRNA for TGF-β RII. The assay used G3PDH, a housekeeping gene, as the marker for quantification. The primer pair used was designed according to the cDNA sequences of mice TGF-β RII (Gene Data Bank) as follows: F: 5'-TCCACCGGCAGCAGAAG-3'; R: 5'-CCCTGTGAACAATGGGCATCT-3' (564 bp). The primer pairs used to amplify G3PDH (452 bp) cDNA were as follows: F: 5'-ACCACAGTCCATGCCATCAC-3'; R: 5'-TCCACCACCCTGTTGCTGTA-3'. The target sequences TGF-β RII and G3PDH were separately amplified *(47)*. Using 2 µL of the first-strand cDNA solution, 50 µL of PCR mixture was prepared containing the following components: 200 m*M* dATP, 200 m*M* dGTP, 200 m*M* dTTP, 200 m*M* dCTP, 20 pmol of the corresponding primer, 1.5 m*M* $MgCl_2$, 20 m*M* Tris buffer (pH 8.3), 50 m*M* KCl, and 1.0 U of *Taq* DNA polymerase (Takara). PCR amplification was performed under the following conditions: denaturation for 1 min, 15 s at 94ºC; annealing for 1 min, 30 s at 60ºC; and polymerization for 2 min at 71ºC. However, the first denaturation step was performed at 94ºC for 5 min, and the final polymerization step was performed for 5 min at 71ºC. PCR amplification was performed for 22, 24, 26, and 28 cycles for TGF-β RII and G3PDH. An aliquot (5 µL) of the PCR-amplified product solution was mixed with an equal volume of the loading solution (50% glycerol, 0.02% bromophenol blue, 0.01 *M* Tris buffer [pH 8.0], 0.1% SDS, and 3 m*M* EDTA). The diluted samples and the sizing ladder (1-kb DNA Ladder; Life Technologies, Gaithersburg, MD) were separated by electrophoresis on 2.0% agarose gel containing ethidium bromide (0.5 mg/mL) at room temperature and 100 V for 20–30 min using Mupid-2 (Cosmo Bio).

After electrophoresis, the bands were visualized with a UV transilluminator and photographed. Band intensities at each number of cycles were plotted on a semilogarithmic graph. For PCR amplification, the initial amount (*A*), efficiency (*R*), amplified amount (*Y*), and the number of cycles (*n*) can be represented as $Y = A(1 + R)n$ *(46)*. The ratio of the initial amount of TGF-β R-II (ARII) to that of G3PDH (AG3) was calculated using the following equation:

$$ARII/AG3 = YRII(1 + RG3)n/YG3(1 + RRII)n$$

R and Y were obtained from the linear portion of the semilogarithmic graph, and the ARII/AG3 ratio, showing the relative amount of TGF-β RII mRNA, was calculated using this equation.

Measurement of Nuclear Protein Concentration

Nuclear extracts of mouse pituitary gland were prepared according to the following method. Pituitary tissue was minced and homogenized with a Wheaton glass grinder in 5 mL of solution containing 0.32 m*M* sucrose, 3 m*M* $MgCl_2$, 40 m*M* KCl, 10 m*M* HEPES (pH 7.5), 1 m*M* diphtheria tetanus toxoid dithiothreitol (DTT), 0.1 m*M* phenylmethylsulfonylfluoride (PMSF), 10 µg of leupeptin, and 2 µg of aprotinin (SMHD buffer) at 4ºC. The homogenate was combined with a 5-mL SMHD rinse of the tissue grinder and centrifuged at 1000*g* for 10 min. The pellet was resuspended in 5 mL of solution containing 0.32 *M* sucrose, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* Tris-HCl (pH 7.5), 1 m*M* DTT, and 0.1 m*M* PMSF (SMCT buffer). Then, 250 µL of 10% (v/v) Nonidet P-40 was added, and the solution was incubated on ice for 10 min and centrifuged at 250*g* for 5 min. The subsequent SMCT wash, nuclear protein extraction by 20 m*M* HEPES (pH 7.8), 0.6 *M* KCl, 0.02 m*M* $ZnCl_2$, 0.2 m*M* EGTA, 0.5 m*M* DTT, 0.1 m*M* PMSF, and 1 µg/µL of pepstatin (buffer II), and centrifugation to obtain the supernatant were identical to the procedures described for the preparation of nuclear extracts from other mouse tissue. The supernatant, containing the high-salt extracts of the nuclei, was dialyzed against 20 m*M* HEPES (pH 7.8), 5 m*M* 2-mercaptoethanol, 50 m*M* NaCl, 2 mM EGTA, 10% (v/v) glycerol, and 0.1 m*M* PMSF and centrifuged at 10,000*g* for 15 min. The protein concentration was determined by the Bradford method using Protein Assay II (Bio-Rad, Hercules, CA).

Western Blotting

Total lysate (50 µg) from the pituitary gland was mixed with an equal volume of sample buffer (0.125 *M* Tris-HCl, pH 6.8; 10% 2-mercaptoethanol; 10% sucrose; 0.004% bromophenol blue) and subjected to SDS-polyacrylamide gel electrophoresis (Mini-PROTEAN; Bio-Rad) on a 7.5–12% gel at 100 V for 1 h and 30 min and transferred onto polyvinylidene difluoride membranes (Bio-Rad) using a trans-blot semidry transfer cell (Trans-Blot SD; Bio-Rad) at 15 V for 30 min. The transferred membrane was soaked in Ponceau-S solution (Sigma) to check the comparable amount of proteins loaded on the gel and the homogeneity of the transfer. The membranes were then blocked using 2% bovine serum albumin in Tween-PBS buffer and incubated at 4ºC overnight with the primary antibodies as follows: anti-p27 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti–cyclin D1 antibody (Santa Cruz), anti-cdk4 antibody (Santa Cruz), and anti-c-*myc* antibody (Santa Cruz). After rinsing three times for 10 min with Tween-PBS, the membranes were incubated with biotinylated anti-IgG at room temperature for 60 min. After rinsing three times for 10 min with Tween-PBS, the membranes were incubated with ABC for 60 min. After rinsing three times for 10 min with Tween-PBS, the membrane was sprayed with DAB-H_2O_2 reaction mixture as chromogenic substrate. The visualized protein bands were analyzed quantitatively by NIH Image software. Protein expression was evaluated as the increase compared to the standard value of wild-type mice.

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