—Original Article—

Characterization of Murine Pituitary-Derived Cell Lines Tpit/F1, Tpit/E and TtT/GF

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Abstract. The pituitary is an important endocrine tissue of the vertebrate that produces and secretes many hormones. Accumulating data suggest that several types of cells compose the pituitary, and there is growing interest in elucidating the origin of these cell types and their roles in pituitary organogenesis. Therein, the histogenous cell line is an extremely valuable experimental tool for investigating the function of derived tissue. In this study, we compared gene expression profiles by microarray analysis and real-time PCR for murine pituitary tumor-derived non-hormone-producing cell lines TtT/GF, Tpit/F1 and Tpit/E. Several genes are characteristically expressed in each cell line: *Abcg2, Nestin, Prrx1, Prrx2, CD34, Eng, Cspg4 (Ng2), S100β* and *nNos* in TtT/GF; *Cxcl12, Raldh1, Msx1* and *Twist1* in Tpit/F1; and *Cxadr, Sox9, Cdh1, EpCAM* and *Krt8* in Tpit/E. Ultimately, we came to the following conclusions: TtT/GF cells show the most differentiated state, and may have some properties of the pituitary vascular endothelial cell and/or pericyte. Tpit/F1 cells show the epithelial and mesenchymal phenotypes with stemness still in a transiting state. Tpit/E cells have a phenotype of epithelial cells and are the most immature cells in the progression of differentiation or in the initial endothelial-mesenchymal transition (EMT). Thus, these three cell lines must be useful model cell lines for investigating pituitary stem/progenitor cells as well as organogenesis.
Key words: Differentiation, Pituitary cell line, Stem/progenitor cell, SOX2

The anterior pituitary (adenohypophysis) is composed of the anterior, intermediate and tuberal lobes and develops from the oral ectoderm by invagination at the early stage of the embryo. During embryonic organogenesis, five types of hormone-producing cells are differentiated to perform essential roles in growth, metabolism, reproduction, lactation, homeostasis and stress response, among others. Many studies hitherto have demonstrated that a number of transcription factors spatiotemporally appear during progression of cell differentiation to produce each hormone-producing cell [1–6]. Stem/progenitor cells definitely differentiate into hormone-producing cells as well as non-endocrine cells and renew themselves as a cell resource ([7] and references therein), but the differentiation mechanism remains to be elucidated. Although recent experiments to form a sphere from the pituitary dispersed cells (pituisphere) have been informative, the heterogeneous population of sphere cells makes it virtually impossible to elucidate the mechanism of differentiation.

Established cell lines are frequently used as model systems not only for cellular physiology and gene expression but also for cell differentiation, providing us with profitable information. The Tpit/F1 cell line was established from the pituitary gland of a temperature-sensitive T antigen transgenic mouse, and it has the characteristics of pituitary S100-positive cells [8]. Mogi *et al.* showed that Tpit/F1 has the ability to differentiate into skeletal muscle cells [9]. On the other hand, TtT/GF was established from a murine thyrotropic pituitary tumor [10], and it has more recently been found to express several stem cell markers [11]. Intriguingly, Tpit/F1 and TtT/GF cells are assumed to be model cells of folliculo-stellate-cells (FS cells), which are candidates for adult pituitary stem/progenitor cells [12, 13]. The remaining non-hormone-producing cell line, Tpit/E cells, is a cell line established in the same experiment as the Tpit/F1 cell line [8], but little is known about its properties. Hence, they might have potential as a pituitary cell resource, but they do not show the same cellular properties [8, 10, 14, 15]. However, further information is required to understand these two cell lines.

In this study, we compared gene expression profiles by microarray analysis and real-time PCR for non-hormone-producing cell lines. Ultimately, the following interpretations were reached: TtT/GF cells are in a mostly but not terminally differentiated state, showing a potency to differentiate into pituitary vascular endothelial cells and/or pericytes. Tpit/F1 show epithelial and mesenchymal phenotypes with stemness still in a transitional state of differentiation, as shown by their expression of *Vimentin* and *Sox2*. Tpit/E cells have a phenotype

Table 1. List of primer sequences for quantitative real-time PCR

Gene	Forward primer	Reverse primer
Sox2	5'-CCGTTTTCGTGGTCTTGTTT-3'	5'-TCAACCTGCATGGACATTTT-3'
Sox9	5'-ACAAGAAAGACCACCCCGATT-3'	5'-GATGTGAGTCTGTTCGGTGGC-3'
Sca1	5'-CCCCTACCCTGATGGAGTCT-3'	5'-GGCAGATGGGTAAGCAAAGA-3'
Cxadr	5'-CCTCTTCTGCTGTCACAGGAA-3'	5'-AGGAACCGCTCCCATTCG-3'
Prop1	5'-CCTTTGGGAGGAACCAGTATC-3'	5'-GCTCTTGCTTCCGTTGCTTA-3'
Prrx1	5'-TTACCCGGATGCTTTTGTTC-3'	5'-ACTTGGCTCTTCGGTTCTGA-3'
Prrx2	5'-AGTGAGGCACGTGTCCAAGTC-3'	5'-GTGGCCAGCATGGCACGTT-3'
Pitx1	5'-GAATCGTCCGACGCTGAT-3'	5'-CGCCGCTGTTTCTTCTTCT-3'
Lhβ	5'-CGGCCTGTCAACGCAACT-3'	5'-GGCAGTACTCGGACCATGCT-3'
Sf1	5'-GGCCGACCAGACCTTTATCT-3'	5'-CAGCTCGCTCCAACAGTTCT-3'
E-cadherin	5'-CCTGCCAATCCTGATGAAAT-3'	5'-CGAACACCAACAGAGAGTCG-3'
Vimentin	5'-TGGTTGACACCCACTCAAAA-3'	5'-GGTCATCGTGATGCTGAGAA-3'
Snail2	5'-TGGCTGCTTCAAGGACACATTAG-3'	5'-CCCTCAGGTTTGATCTGTCTGC-3'
S100β	5'-ACGAGCTCTCTCACTTCCTGGA-3'	5'-AGTCACACTCCCCATCCCC-3'
T-antigen	5'-AGAATGGATGGCTGGAGTTGC-3'	5'-TTTCTCGAGTTATGTTTCAGGTTCAGGGGG-3'
Tbp	5'-GATCAAACCCAGAATTGTTCTCC-3'	5'-ATGTGGTCTTCCTGAATCCC-3'

of epithelial cells, and they are the most immature cells with abundant expression of the stem cell marker *Sox2*; they are probably in the process of differentiating or in the initial endothelial-mesenchymal transition (EMT) expressing *Sox9* and *Snail2* (*Slug*). Thus, these three cell lines will surely be useful model cells for investigating pituitary stem/progenitor cells as well as organogenesis.

Materials and Methods

Cell culture, cell counting and total RNA preparation

Tpit/E, TpitF1 and TtT/GF cell lines were kindly provided to us by Professor K Inoue (Saitama University). Culture of the cell lines was carried out in a humidified atmosphere of 5% CO_2 and 95% air. Tpit/E and Tpit/F1 were maintained in a mixed medium comprised of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) and Ham's F-12 (1:1; Invitrogen) supplemented with 10% horse serum and 2.5% fetal bovine serum (FBS) at 33 C. TtT/GF cells were maintained in DMEM supplemented with 10% FBS at 37 C. Growth curves of Tpit/E, Tpit/F1 and TtT/GF were described by counting the cells seeded and cultured on 24-well plates (Corning, NY, USA) at 75,000, 37,500 and 12,500 cells per well, respectively. Total RNAs were prepared from each cell line using ISOGEN (Nippon Gene, Tokyo, Japan).

Microarray analysis

Microarrays were performed using Mouse Genome 430 2.0 GeneChips (Affymetrix Japan, Tokyo, Japan) for total RNA samples from TtT/GF, Tpit/F1 and Tpit/E. Data of microarrays were normalized by median normalization.

Quantitative real-time PCR

Reverse transcripts were synthesized with PrimeScript Reverse Transcriptase (Takara Bio, Kyoto, Japan) using 1 μg of total RNA after DNase I treatment and were subjected to quantitative real-time PCR on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems,

Foster City, CA, USA) with SYBR Green Realtime PCR Master Mix Plus (Toyobo, Osaka, Japan) and specific primer sets (Table 1) at 0.6 μM for each target gene. Each sample was measured in duplicate in two independent experiments, and data were calculated by the comparative C_T method (DDC_T method) to estimate the gene copy number relative to TATA box binding protein (*Tbp*) as an internal standard. The DNA sequence of the PCR product of each sample was confirmed by nucleotide sequencing (data not shown).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 20 mM HEPES, pH 7.5, for SOX2 staining or with 95% ethyl alcohol for E-cadherin staining for 30 min at room temperature, followed by a wash with 20 mM HEPES-100 mM NaCl and by incubation with 20 mM HEPES, pH 7.5, containing 10% (v/v) fetal bovine serum, 100 mM NaCl and 0.4% (v/v) Triton X100 (blocking buffer) for 60 min at room temperature. Reaction with primary antibodies was performed at an appropriate dilution with blocking buffer overnight at 4 C. The primary antibodies used were goat IgG against human SOX2 (1:500 dilution; Neuromics, Edina, MN, USA), mouse IgG against human E-cadherin (1:200 dilution; BD Biosciences, San Jose, CA, USA) and guinea pig antiserum against ovine LHβ (1:8,000 dilution), which was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) through the courtesy of Dr. AF Parlow. After washing, cells were reacted with secondary antibodies using Cy3-conjugated AffiniPure donkey anti-goat and mouse IgG (1:500 dilution; Jackson ImmunoResearch, West Grove, PA, USA). Cells were washed and then incubated in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA) with DAPI (4,6'-diamidino-2-phenylindole, dihydrochloride). Immunofluorescence was observed under fluorescence microscopy with a DM16000 B inverted microscope (Leica, Wetzlar, Germany).

Results

Cell appearance and growth of TtT/GF, TpitF1 and Tpit/E cells

To characterize the three non-hormone-producing pituitary cell lines, we first observed the cell appearance and proliferative activity. TtT/GF cells showed a thin, flat star-like appearance with long multipolar cytoplasmic processes like a capillary (Fig. 1A), as reported previously [10]. Tpit/F1 cells showed a star-like appearance [8] as with TtT/GF cells, but they had shorter cytoplasmic processes, and more of them, than TtT/GF cells (Fig. 1B). These common characteristics of the many spreading multipolar processes of TtT/GF and Tpit/F1 cells show their mesenchymal phenotype. The remaining non-hormone-producing cell line, Tpit/E cells, is a cell line established in the same experiment as the Tpit/F1 cell line from an anterior pituitary gland of a temperature-sensitive large T antigen (*T-antigen*) transgenic mouse [8], but it has not been well characterized so far. The appearance of Tpit/E cells is clearly different from those of TtT/GF and Tpit/F1 cells (Fig. 1C). Tpit/E cells are polygonal in shape, are very densely packed together and grow like blocks in a wall, showing epithelial phenotypes dissimilar to Tpit/F1 and TtT/GF cells.

When cell growth was measured for five or seven days, Tpit/F1 cells showed slower growth profiles than those of TtT/GF and Tpit/E cells (Fig. 1). The doubling times of the TtT/GF, Tpit/F1 and Tpit/E cells were different from each other, about 14.4, 31.6 and 28.6 h, respectively, showing the unique origins of the three non-hormone-producing cell lines.

Expression profile of genes in Tpit/E, Tpit/F1 and TtT/GF cells

To characterize Tpit/E, Tpit/F1 and TtT/GF cells, we performed microarray analyses. After normalizing the signals by median normalization, comparison of the characteristically expressing genes was performed among the three cell lines in the following categories: stem/progenitor cell, transcription factor participating in early pituitary development, committed and/or terminally differentiated pituitary cells, epithelial/mesenchymal cells including the epithelial-mesenchymal transition (EMT), angiogenesis/endothelial cells/pericytes and others (Table 2).

The comparison of microarray data made it possible to distinguish among the expressions of several genes in a particular cell line, as shown in Table 2. Subsequently, real-time PCR was examined to confirm the expression of some distinctive genes, in addition to genes of interest in our recent studies on pituitary organogenesis, such as *Sox2, Sca1, Prop1, Prrx1, Prrx2* and *Snail2* (asterisks in Table 2). In Fig. 2, data from quantitative real-time PCR are presented as amounts relative to those of TATA-box binding protein (*Tbp*). Microarray and real-time PCR are based on different detection principles, hybridization and amplification, which depend on the Tm value and compatibility of the primer pair, respectively. The values are not necessarily the same, but most of the genes examined showed roughly similar expression profiles to those examined by microarray (Fig. 2 and Table 2), while real-time PCR showed small differences in values for *Prrx2, Pitx1, Vimentin* and *Snail2* in comparison with those obtained by microarray.
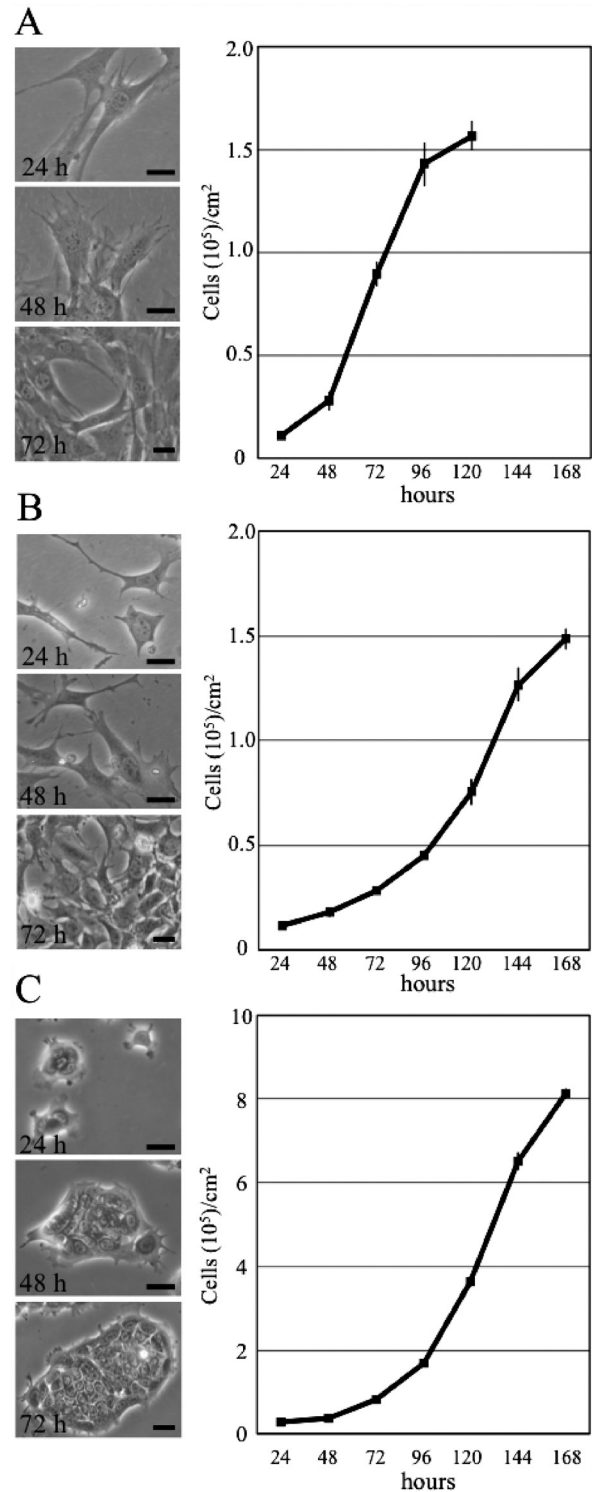

Fig. 1. Cell appearance and growth curves for TtT/GF, Tpit/F1 and Tpit/E cells. Morphology by light microscopy (left panels) and growth curves (right panels) for TtT/GF (A), Tpit/F1 (B) and Tpit/E cells (C) are indicated. Cell numbers of each line were counted during the period of 24 to 120 h or that of 24 to 168 h, and the doubling times of TtT/GF, Tpit/F1 and Tpit/E were calculated with the cell numbers for 48 to 72 h, 120 to 144 h and 120 to 144 h, respectively. The data are presented as the mean ± SD of duplicate counts in two independent experiments. Scale bar= 20 μm.

Table 2.   List of expressing genes characteristic of the Tpit/E, Tpit/F1 and TtT/GF cells

Gene title	Gene symbol	Ratio per each median			Accession number
		Tpit/E	Tpit/F1	TtT/GF	
Stem/progenitor cells					
ATP-binding cassette, sub-family G (WHITE), member 2	*Abcg2 (Bcrp1)*	0	0	17	NM_011920
* Coxsackie virus and adenovirus receptor	*Cxadr (Car)*	20	1	1	NM_001025192
Chemokine (C-X-C motif) ligand 12	*Cxcl12*	0	344	7	NM_001012477
Chemokine (C-X-C motif) receptor 4	*Cxcr4*	0	1	0	NM_0099113
Glial cell line derived neurotrophic factor family receptor alpha 2	*GFRa2*	1	1	0	NM_008115
Kruppel-like factor 4 (gut)	*Klf4*	2024	973	155	NM_010637
Nanog homeobox	*Nanog*	0	1	0	NM_028016
Nestin	*Nestin*	2	3	49	NM_016701
* Paired like homeodomain factor 1	*Prop1*	1	1	1	NM_008936
* Paired related homeobox 1	*Prrx1*	0	1	16	NM_001025570
* Paired related homeobox 2	*Prrx2*	0	10	40	NM_009116
Aldehyde dehydrogenase family 1, subfamily A1	*Raldh1*	21	199	18	NM_013467
Aldehyde dehydrogenase family 1, subfamily A2	*Raldh2*	0	0	0	NM_009022
* Lymphocyte antigen 6 complex, locus A	*Sca1*	622	92	263	NM_010738
* SRY-box containing gene 2	*Sox2*	61	12	7	NM_011443
* SRY-box containing gene 9	*Sox9*	155	16	2	NM_011448
Transcription factors participating in early pituitary development					
Homeobox gene expressed in ES cells	*Hesx1*	0	0	0	NM_010420
ISL1 transcription factor, LIM/homeodomain	*Isl1*	24	25	17	NM_021459
LIM homeobox protein 3	*Lhx3*	0	0	1	NM_001039653
LIM homeobox protein 4	*Lhx4*	0	1	0	NM_010712
Homeobox, msh-like 1	*Msx1*	2	21	3	NM_010835
Orthodenticle homolog 2 (Drosophila)	*Otx2*	0	0	0	NM_144841
Paired box gene 6	*Pax6*	0	0	2	NM_013627
* Paired-like homeodomain transcription factor 1	*Pitx1*	1878	785	8	NM_011097
Paired-like homeodomain transcription factor 2	*Pitx2*	89	150	9	NM_001042502
Sine oculis-related homeobox 1	*Six1*	26	133	7	NM_009189
Sine oculis-related homeobox 6	*Six6*	0	0	0	NM_011384
Committed and/or terminally differentiated pituitary cells					
Pro-opiomelanocortin-alpha	*Acth*	0	0	2	NM_001278581
Follicle stimulating hormone beta	*Fshβ*	0	0	0	NM_008045
GATA binding protein 2	*Gata2*	0	19	2	NM_008090
Growth hormone	*Gh*	0	0	1	NM_008117
* Luteinizing hormone beta	*Lhβ*	10	6	0	NM_008497
Cadherin 2	*N-cadherin*	0	6	49	NM_007664
Neurogenic differentiation 1	*NeuroD1*	0	0	1	NM_010894
POU domain, class 1, transcription factor 1	*Pit1*	0	0	1	NM_008849
Prolactin	*Prl*	0	0	0	NM_001163530
* Nuclear receptor subfamily 5, group A, member 1	*Sf1 (Nr5a1)*	7	5	0	NM_139051
T-box 19	*Tbx19*	0	0	1	NM_032005
Thyroid stimulating hormone, beta subunit	*Tshβ*	0	0	1	NM_001165939
Epithelial/Mesenchymal cells including EMT					
* Cadherin 1	*Cdh1 (E-cadherin)*	291	1	0	NM_009864
Epithelial cell adhesion molecule	*EpCAM*	193	0	5	NM_008532
Keratin 8	*Krt8*	538	1	2	NM_031170
Matrix metallopeptidase 14 (membrane-inserted)	*Mmp14*	432	1056	75	NM_008608
Snail homolog 1 (Drosophila)	*Snail1*	0	0	1	NM_011427
* Snail homolog 2 (Drosophila)	*Snail2 (Slug)*	545	548	82	NM_011415
Transforming growth factor, beta receptor II	*Tgfbr2*	509	1135	47	NM_009371
Twist basic helix-loop-helix transcription factor 1	*Twist1*	5	23	8	NM_011658
Twist basic helix-loop-helix transcription factor 2	*Twist2*	0	25	12	NM_007855
* Vimentin	*Vimentin*	5	1658	713	NM_011701
Angiogenesis/Endothelial cells/Pericyte					
CD34 antigen	*CD34*	0	3	112	NM_133654
Actin, alpha 2, smooth muscle, aorta	*Acta2 (αSMA)*	1399	6362	467	NM_007392
Endoglin	*Eng*	2	4	13	NM_007932
Fibronectin 1	*Fn1*	60	238	236	NM_010233
Endothelial-specific receptor tyrosine kinase	*Tek*	0	0	0	NM_013690
Kinase insert domain protein receptor	*Kdr (Flk1)*	0	2	1	NM_010612
Cadherin 5 (VE-Cadherin)	*Cdh5*	0	1	1	NM_009868
Platelet/endothelial cell adhesion molecule 1	*Pecam1*	0	0	0	NM_001032378
Desmin	*Des*	0	1	3	NM_010043
Chondroitin sulfate proteoglycan 4	*Cspg4 (Ng2)*	0	1	19	NM_139001
Platelet derived growth factor receptor, beta polypeptide	*Pdgfrb*	0	343	96	NM_008809
Regulator of G-protein signaling 5	*Rgs5*	0	0	1	NM_009063
Others					
Interleukin 6	*IL6*	0	2	0	NM_031168
* S100 protein, beta polypeptide, neural	*S100β*	1	2	18	NM_009115
nitric oxide synthase 1, neuronal	*nNos*	0	4	12	NM_008712

The signal value was normalized by median normalization. Asterisks (*) indicate genes analyzed by quantitative real-time PCR.
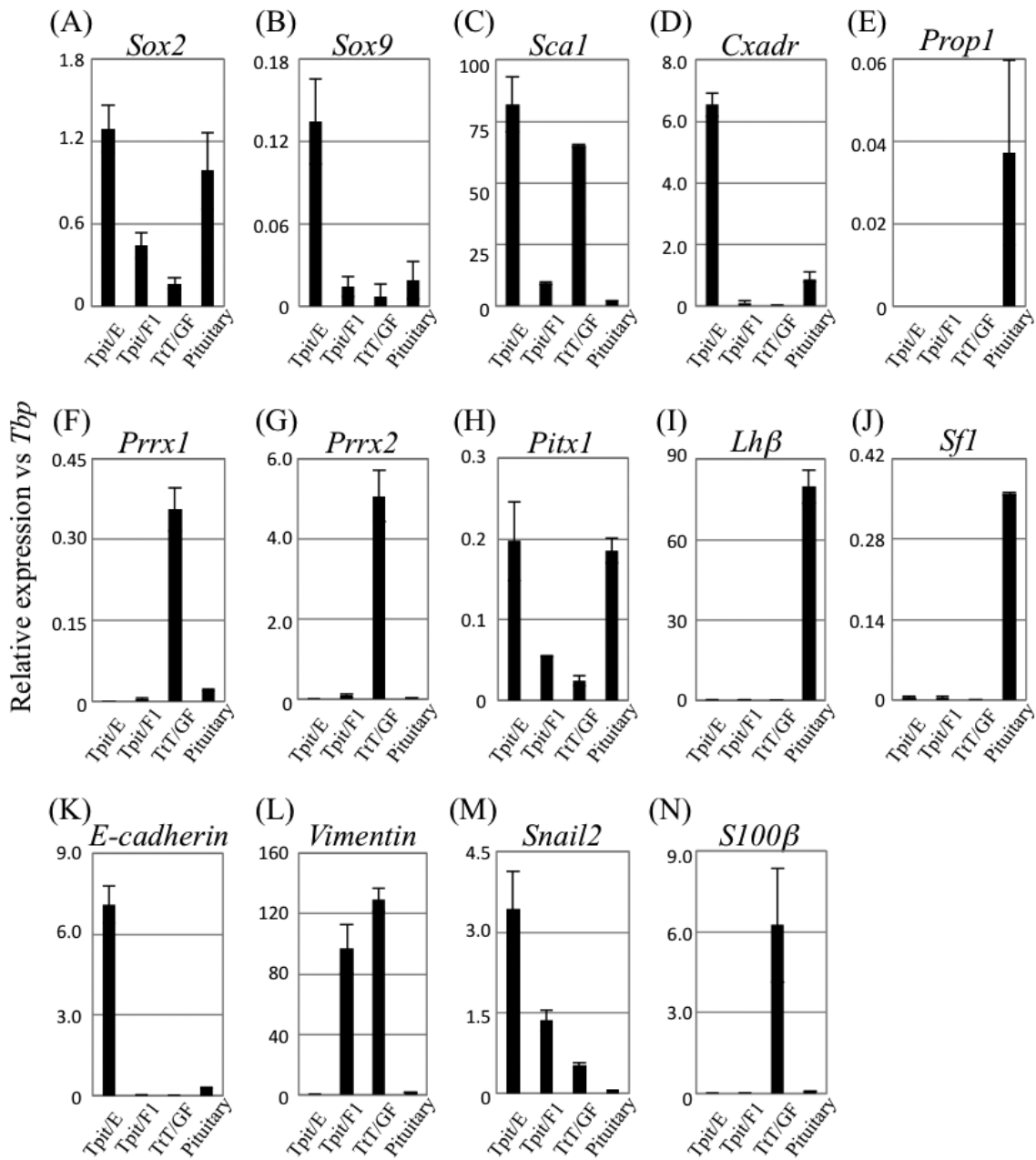
Fig. 2. Real-time PCR of genes of interest expressing in Tpit/E, TpitF1 and TtT/GF cells. Quantitative real-time PCR was performed to estimate the mRNA level of the following genes: *Sox2* (A), *Sox9* (B), *Sca1* (C), *Cxadr* (D), *Prop1* (E), *Prrx1* (F), *Prrx2* (G), *Pitx1* (H), *Lhβ* (I), *Sf1* (J), *E-cadherin* (K), *Vimentin* (L), *Snail2* (M) and *S100β* (N). Data were calculated by the comparative CT method to estimate the relative copy number contrasted to that of the TATA box binding protein gene (*Tbp*) used as an internal standard. The data are presented as the ± SD of duplicate PCRs in two independent experiments.

Stemness of Tpit/E, TpitF1 and TtT/GF cells

Hitherto, the differentiation potency of Tpit/F1 cells [9] and expression of stem/progenitor markers in TtT/GF cells [11] have been reported. To determine the stemness of the cell lines, we first verified the expression of a stem/progenitor marker, *Sox2*. As shown in Fig. 2A and Table 2, all three cell lines expressed *Sox2,* with the order from highest to lowest being Tpit/E, Tpit/F1 and TtT/GF

cells. Immunocytochemistry demonstrated that SOX2 signals were strongly detected in Tpit/E cells (Fig. 3A). Notably, very weak positive cells were scattered in the other two lines (Fig. 3A), indicating that these cell lines are heterogeneous. *Sox9* is known to play a role in progenitor cells in a committed and/or progressing state [16, 17]. *Sox9* expression was observed abundantly in Tpit/E cells, while the other two lines had very low amounts (Fig. 2B). We subsequently
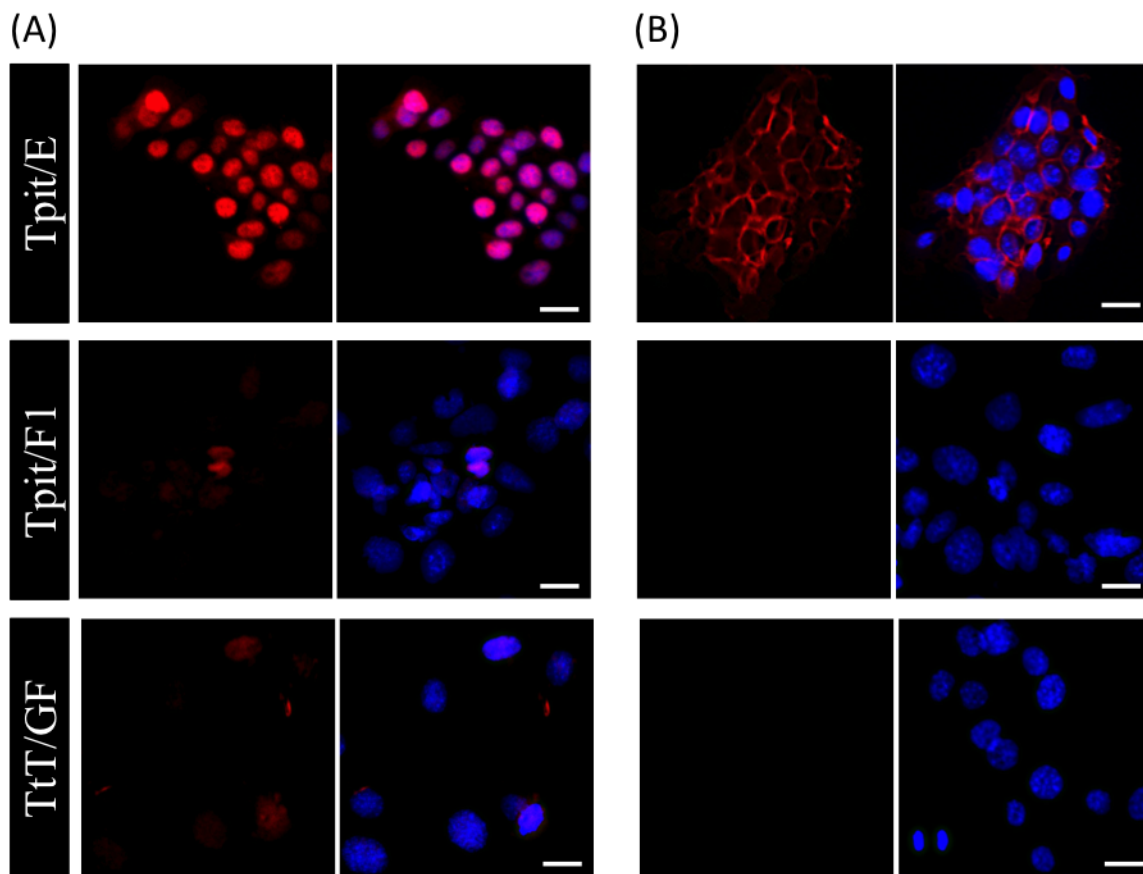
Fig. 3. Immunocytochemistry for SOX2 and E-cadherin in Tpit/E, Tpit/F1 and TtT/GF cells. Immunostaining (red) for SOX2 (A) and E-cadherin (B) was performed and merged with an image of nuclear staining with DAPI (blue) (each right panel). Scale bar= 20 μm.

verified the expression of *Sca1* (stem cell antigen 1), which is known to be associated with activity in stem and progenitor cells [18] and is present in the endothelial phenotype subset (Sca1^{high}-SP cells) of the side population (SP) [19]. As shown in Fig. 2C, *Sca1* was expressed in all three cell lines, with especially high levels in Tpit/E (at about 80-fold/*Tbp*) and TtT/GF (at about 65-fold/*Tbp*), respectively, and with a higher level in Tpit/F1 cells (about 10-fold/*Tbp*) than in the pituitary (about 2-fold/*Tbp*).

More recently, CAR (coxsackievirus and adenovirus receptor encoding by *Cxadr*) was found in SOX2/E-cadherin double-positive pituitary stem/progenitor cells forming a niche [20]. As shown in Fig. 2D, *Cxadr* was expressed in Tpit/E cells but not in Tpit/F1 and TtT/GF cells.

Our recent studies revealed that *Prop1*, *Prrx1* and *Prrx2* play crucial roles in pituitary stem/progenitor cells [20–25]. Although the pituitary-specific transcription factor *Prop1* was not expressed in any cell lines (Fig. 2E), the mesenchymal markers *Prrx1* and *Prrx2* were expressed mainly in TtT/GF, with a small amount in Tpit/F1 cells as shown in Figs. 2F and G, respectively. In addition, microarray analysis showed that expression of *Cxcl12* and *Raldh1* in Tpit/F1 cells and *Nestin* in TtT/GF cells was prominent (Table 2).

Early pituitary transcription factors of Tpit/E, TpitF1 and TtT/ GF cells

Among the early pituitary transcription factors, we performed real-time PCR for *Pitx1*, which functions as a pan-pituitary transcription factor [26] and as a crucial factor for development of the brain, face and hind limb [27]. The highest expression of *Pitx1* was observed in Tpit/E cells, and the amount was similar to that in the pituitary (Fig. 2H). Although the microarray data showed a very high median value for *Pitx1,* at 1878 and 785 in Tpit/E and Tpit/F1 cells, respectively, the value from the real-time PCR was very low, at about 0.2-fold/*Tbp*, reflecting the difference between the two methods. *Pitx2*, a cognate of *Pitx1*, also showed a high value in Tpit/E and Tpit/F1 cells (Table 2). MSX1 and SIX1 play roles in the cranial placode and contribute to development of the pituitary gland [28]. Microarray data showed that *Msx1* and *Six1* were expressed at a relatively higher level in Tpit/F1 than in the other two cell lines (Table 2).

Differentiation markers of Tpit/E, TpitF1 and TtT/GF cells

N-cadherin, a marker of differentiated cells in the pituitary [29], is known to play a role in cell migration essential for embryonic development and the epithelial-mesenchymal transition (EMT) [30].

As shown in Table 2, *N-cadherin* is expressed in TtT/GF cells, and although a low amount of *Lhβ* and *Sf1* expression was observed by microarray analysis (Table 2), we confirmed that they were not found in any of the three lines by real-time PCR (Fig. 2I and J). Immunocytochemistry for LHβ in the three cell lines was also negative (data not shown).

Epithelial/mesenchymal cell and EMT markers of Tpit/E, TpitF1 and TtT/GF cells

Markers of this category showed unique expressions in the three cell lines. Tpit/E cells are specifically expressed cell adhesion proteins, *E-cadherin* and *EpCAM*, and intermediate filament *Krt8* (Fig. 2K, Table 2 and Fig. 3B). They are known as epithelial cell markers, and *E-cadherin* and *Krt8* are expressed in pituitary stem/progenitor cells [30–33]. On the other hand, *Vimentin*, a marker of mesenchymal cells and EMT [34], was dominantly detected in both Tpit/F1 and TtT/GF cells (Fig. 2L). Notably, *Mmp14* (membrane-bound protease), *Snail2* (same as *Slug*, repressor for E-cadherin expression and an inducer of EMT) and *Tgfbr2* (TGFβ receptor 2), which are known to be involved in transition and migration of cells during the EMT [35–37], were expressed in the three cell lines, although their quantities differed (Fig. 2M and Table 2).

Angiogenesis/endothelial/pericyte cell markers and other genes of Tpit/E, TpitF1 and TtT/GF cells

CD34, *Acta2* and *Fn1* are known to be characteristic in vascular/endothelial cells. *CD34* was remarkably expressed in TtT/GF, while *Acta2* and *Fn1* were expressed in all three cell lines (Table 2). *Cspg4* (*Ng2*), which is a pericyte marker, was expressed in TtT/GF cells but not in Tpit/E and Tpit/F1 cells. Another pericyte marker, *Pdgfrb*, was expressed in TtT/GF and Tpit/F1 cells. *S100β*-expressing cells are known to be composed of heterogeneous subpopulations in view of their morphologies and components and to show versatile biological roles [12, 13]. Real-time PCR revealed that *S100β* was predominantly expressed in TtT/GF cells, the unique characteristics of which have been reported previously (Fig. 2N) [11].

Expression of T-antigen in Tpit/E, TpitF1 and TtT/GF cell lines

Tpit/E and Tpit/F1 cell lines were established from an anterior pituitary gland of a temperature-sensitive large *T-antigen* transgenic mouse [8]. We examined the expression levels of *T-antigen* by real-time PCR and demonstrated that this gene is expressed in Tpit/F1 cells but not in Tpit/E cells, suggesting the possibility of a self-renewal ability independent of T-antigen (Fig. 4).

Discussion

This study analyzed the profile of gene expression in three dissimilar murine pituitary-derived non-endocrine cell lines as well as their phenotypes. By comparing gene expression profiles and overviews among the three cell lines, we summarized the characteristic genes of each cell line, as shown in Table 3. From the results, we considered the properties of these three cell lines to be as follows.

TtT/GF cells distinctively express *Vimentin, Prrx1, Prrx2, Nestin* and *CD34* and *N-cadherin* but not *E-cadherin,* in addition to a low
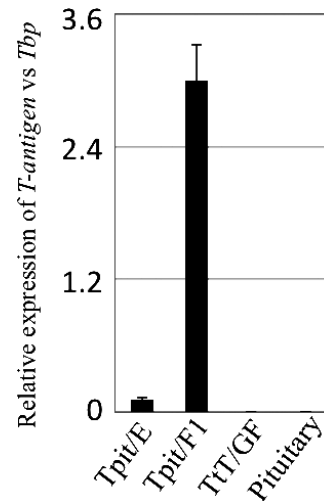

Fig. 4. Real-time PCR of *T-antigen* in Tpit/E, TpitF1 and TtT/GF cells. Quantitative real-time PCR was performed to estimate the mRNA level of *T-antigen* in Tpit/E, TpitF1 and TtT/GF cells. Data were calculated as in Fig. 2.

level of expression of *Sox2*. Taking the gene expression profiles together with the cell morphology, TtT/GF cells have a mesenchymal phenotype and are in the most differentiated state, but not terminally differentiated, among the three cell lines. Recently, we demonstrated that the pituitary contains two types of PRRX1- and PRRX2-positive cells that originate from the pituitary of the ectoderm and from the mesenchyme of the surrounding extrapituitary cells [25]. In addition, NESTIN-positive cells were observed as mesenchymal cells invading from surrounding extrapituitary cells during the embryonic period (unpublished data) and dividing progenitor/commitment cells [38]. TtT/GF, as well as Tpit/E and Tpit/F1, expresses *Sca1*, which is expressed in the endothelial phenotype subset (Sca1^{high}-SP cells). Taken together with the expression of *CD34, Cspg4* (*Ng2*) and other genes described below, TtT/GF cells likely have a non-pituitary origin and may have some properties of pituitary vascular endothelial cells and/or pericytes rather than model cells of FS cells, as described previously [11].

Tpit/E cells, which express *E-cadherin, EpCAM* and *Krt8,* show a phenotype close to that of epithelial cells. Considering the expression of *Snail2* (*Slug*), Tpit/E is still in the process of differentiation or initial EMT. Tpit/E cells also express *Sox2* and *Sox9* at high levels, indicating that this cell line has the most stemness among the three cell lines. T-antigen-independent proliferation of Tpit/E may indicate that Tpit/E cells have an ability to self-renew and may be a non-tumorous pituitary stem/progenitor cell line. Furthermore, this cell line expresses *Cxadr* (CAR), which is present in the SOX2/E-cadherin double-positive cells on the marginal cell layer (MCL) facing Rathke's residual lumen and in the SOX2-positive cells scattered in the parenchyma as a cluster, forming a pituitary stem/progenitor niche [20]. Notably, we simultaneously found that CAR-positive pituitary stem/progenitor cells migrate to the parenchyma by EMT with transient expression of *Vimentin* and form a parenchymal niche comprising a SOX2-positive cluster by MET [20]. Taken together

Table 3. Comparison of the characteristic genes in Tpit/E, Tpit/F1 and TtT/GF cells

Category	Tpit/E Tpit/F1 TtT/GF	Tpit/E	Tpit/E Tpit/F1	Tpit/F1	Tpit/F1 TtT/GF	TtT/GF	Tpit/E TtT/GF	N/A
Stem/progenitor cells	*Klf4, Sca1, Sox2*	*Cxadr (Car), Sox9*		*Cxcl12, Raldh1*	*Abcg2 (Bcrp1), Nestin, Prrx1, Prrx2*			*Cxcr4, GFRa2, Nanog, Prop1, Raldh2*
Transcription factors participating in early pituitary development	*Isl1*		*Pitx1, Pitx2, Six1*	*Msx1*				*Hesx1, Lhx3, Lhx4, Otx2, Pax6, Six6*
Committed and/or terminally differentiated pituitary cells						*N-cadherin*		*Acth, Fshβ, Gata2, Gh, Lhβ, NeuroD1, Pit1, Prl, Sf1 (Nr5a1), Tbx19, Tshβ*
Epithelial/mesenchymal cells including EMT — Epithelial		*Cdh1 (E-cadherin), EpCAM, Krt8*						
Epithelial/mesenchymal cells including EMT — Mesenchymal					*Vimentin*			
Epithelial/mesenchymal cells including EMT — EMT	*Snail2 (Slug)*		*Mmp14, Tgfbr2*	*Twist1*	*Twist2*			*Snail1*
Angiogenesis/endothelial cells/pericytes	*Acta2 (αSMA), Fn1*				*Pdgfrb*	*CD34, Eng, Cspg4 (Ng2)*		*Tek, Kdr (Flk1), Cdh5, Pecam1, Des, Rgs5*
Others						*S100β, nNos*		*IL6*

Characteristic and common genes of Tpit/E, Tpit/F1 and TtT/GF cells are listed. N/A: not applicable to the three cell lines.

with the expression of *Pitx1*, these data suggest that the Tpit/E cell line will surely be a useful model cell for investigating pituitary stem/progenitor cells and niches, as well as pituitary organogenesis.

Tpit/F1 cells express not only epithelial factor genes, *Pitx1* and *Sox2*, but also a mesenchymal marker, *Vimentin*. This profile shows epithelial and mesenchymal phenotypes as well as stemness, indicating a cell line in a transiting state of differentiation. We observed similar cells positive for SOX2 and Vimentin in the adult rat pituitary (data not shown). The expression of *Msx1*, *Cxcl12* and *Raldh1* was notable. Although *Msx1* is a mesenchymal factor, it plays a role in the pituitary [39, 40]. *Cxcl12*, which plays an important role in the hematopoietic stem cell, was revealed to be specifically expressed in the non-endocrine S100-positive cells in the pituitary [41]. In addition, *Raldh1* produces retinoic acid, which is involved in embryonic development, and shows tissue-specific expression [42], and it has been found in S100-positive cells and prolactin-producing endocrine cells [43, 44]. Meanwhile, Tpit/F1 was found to have the ability to differentiate into skeletal muscle cells [9]. Taken together, Tipt/F1 cells may be responsible for several stimuli of differentiation.

Thus, the TtT/GF, Tpit/E and Tpit/F1 cell lines might be progenitor cells that are able to differentiate. Despite their cell lineages, these cell lines must be valuable tools for the study of pituitary organogenesis. Hereafter, it will be interesting to examine their potencies for differentiation into hormone producing cells, especially in the case of Tpit/E and Tpit/F1. Approaches to induce differentiation will surely provide us with valuable findings regarding molecular mechanisms for differentiation and supply systems of hormone-producing cells in the pituitary.

Acknowledgments

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