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REGULAR ARTICLE

# Characterization of a pituitary-tumor-derived cell line, TtT/GF, that expresses Hoechst efflux ABC transporter subfamily G2 and stem cell antigen 1

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**Abstract** The anterior lobe of the pituitary gland is composed of five types of endocrine cells and of non-endocrine folliculo-stellate cells that produce various local signaling molecules. The TtT/GF cell line is derived from pituitary tumors, produces no hormones and has folliculo-stellate cell-like characteristics. The biological function of TtT/GF cells remains elusive but several properties have been postulated (support of endocrine cells, control of cell proliferation, scavenger function). Recently, we observed that TtT/GF cells have high resistance to the antibiotic G418 and low influx for Hoechst 33342, indicating the presence of ATP-binding cassette (ABC) transporters that efflux multiple drugs, i.e., a property similar to that of stem/-progenitor cells. Therefore, we examine TtT/GF cells for the presence of ABC transporters, for the efflux ability of Hoechst 33342 and for those genes characteristic of TtT/GF cells. Real-time polymerase chain reaction (PCR) for ABC transporters demonstrated that *Abcb1a*, *Abcb1b* and *Abcg2*, regarded as stem cell markers, were characteristically expressed in TtT/GF cells but not in Tpit/F1 and LβT2 cells. Furthermore, the remarkable low-efflux ability of Hoechst 33342 from TtT/GF cells was confirmed by using inhibitors and contrasted with the abilities of Tpit/F1 and LβT2 cells. The high and specific expression of stem cell antigen 1 (*Sca1*) in TtT/GF cells was confirmed by real-time PCR. We also demonstrated those genes that are expressed abundantly and characteristically in TtT/GF, suggesting that TtT/GF cells have unique characteristics similar to those of stem/progenitor cells of endothelial or mesenchymal origin. Thus, the present study has revealed an intriguing property of TtT/GF cells, providing a new clue for an understanding of the function of this cell line.

**Keywords** TtT/GF cell line · ABC transporter · Sca1 · Hoechst 33342 · Stem cell

Hideo Mitsuishi and Takako Kato contributed equally to this work.

The authors have no competing interests to declare

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## Introduction

The pituitary non-endocrine cell has received attention as a key cell involved in hormone-producing cell renewal, since several recent studies have demonstrated that the pituitary contains stem/progenitor cells (Allaerts and Vankelecom 2005; Osuna et al. 2012; Vankelecom 2010, 2012; Yoshida et al. 2009, 2011). Folliculo-stellate cells in the pituitary are known as a major type of non-endocrine cell and are considered to be a resource for cell renewal (Devnath and Inoue 2008; Inoue et al. 2002; Vankelecom 2007). Hence, non-endocrine cell lines derived from pituitary tumors are useful tools for investigating the cell renewal system of this tissue. TtT/GF is a cell line established from mouse pituitary tumors by induction with radiothyroidectomy (Inoue et al. 1992) and is defined as a non-endocrine cell expressing S100 and glial fibrillary acidic

protein (GFAP), which are characteristics of pituitary folliculo-stellate cells. To date, many investigators have investigated TtT/GF cells in order to reveal their characteristics and have reported several feasible activities (Jin et al. 2000; Lohrer et al. 2001; Perez Castro et al. 2000; Renner et al. 1997; Traverso et al. 1999; Yamasaki et al. 1997; Zhang et al. 1997). Nevertheless, the definitive role of the TtT/GF cell is still unclear.

Recently, using TtT/GF cells, we examined a stable transfection of expression vector and observed an extraordinary resistance to the antibiotic geneticin (G418) in the selection of transformed cells. From this observation, we hypothesized that TtT/GF cells have efficient activities for drug efflux, as exhibited by their ATP-binding cassette transporters (ABC transporter). We were thus provided with a novel perspective concerning the properties of the TtT/GF cell in comparison with those of Tpit/F1 and LβT2 cells, which are non-endocrine folliculo-stellate-like cell lines with different properties from those of TtT/GF and gonadotrope-lineage cell lines, respectively, generated separately from mouse pituitary tumors. Notably, stem/progenitor cells, which participate in cell renewal, are known to have efficient efflux activity for Hoechst 33342 through a multidrug transporter, namely ABC transporter superfamily G member 2 (ABCG2; van Veen et al. 2001; Zhou et al. 2001). Based on a report suggesting the presence of ABC transporters in TtT/GF cells (Chapman et al. 2002), we have characterized further ABC multidrug transporters in these cells. Finally, we have demonstrated that TtT/GF cells have the distinct multidrug transporter ABCG2 plus ABCB1a, ABCB1b and ABCC1. Additionally, we have observed transcripts of *Sca1* (*stem cell antigen 1*) and other characteristic genes in the TtT/GF cells. The present study has therefore revealed a novel property of TtT/GF cells.

## Materials and methods

### Materials

DMEM (Dulbecco's Modified Eagle Medium) and Ham's F-12 were obtained from Invitrogen (Carlsbad, Calif., USA). Mouse pituitary tumor cell line LβT2 (Alarid et al. 1996), which expresses pituitary glycoproteins α and luteinizing hormone β, was kindly supplied by Dr. P. L. Mellon. Two non-endocrine cell lines, namely TtT/GF (established from mouse tumor by radiothyroidectomy; Inoue et al. 1992) and Tpit/F1 (established from mouse tumor by expression of temperature-sensitive mutant T-antigen; Chen et al. 2000), were supplied by Dr. K. Inoue. Hoechst 33342, G418, methyl thiazolyl tetrazolium (MTT), and verapamil were purchased from Sigma-Aldrich (St. Louis, Mo., USA). MK571 was from Calbiochem (San Diego, Calif., USA). For the polymerase chain reaction (PCR), the SYBR Green Real-time PCR Master Mix was purchased from Toyobo (Osaka, Japan).

### Cell culture

Tpit/F1 cells were maintained in a mixed medium comprising DMEM and Ham's F-12 (1:1) with 10 % horse serum and 2.5 % fetal bovine serum (FBS) at 33 °C and TtT/GF and LβT2 cells were kept in DMEM supplemented with 10 % FBS at 33 °C in a humidified atmosphere of 5 % $CO_2$ and 95 % air. The trypsin-dispersed cells were seeded on 96-well plates at $1 \times 10^4$ cells/100 μl per well. All cell lines were kept in culture for no longer than eight passages.

### Measurement of cell survival rate against G418

Cell survival rate was examined at 100–2,000 μg/ml in G418 culture media for 3 days and was measured colorimetrically with the MTT assay (Mosmann 1983). Briefly, MTT (0.1 mg in 100 μl) was added and incubated at 37 °C for 3 h. After removal of the media and addition of 100 μl dimethyl sulfoxide to dissolve the dark blue crystals, absorption at 570 nm was measured with a Wallac 1420 ARVOsx Multilabel Counter (PerkinElmer Life Sciences, Waltham, Mass., USA)

### Measurement of Hoechst 33342 efflux

Verapamil (an inhibitor of ABCB1a, ABCB1b and ABCG2 and a blocker of the L-type calcium channel) was dissolved in water and used at 50 and 100 μM and MK571 (an inhibitor of ABCB1, ABCB2, ABCG2 and ABCC1) was dissolved in phosphate-buffered saline and used at 40 and 80 μM, respectively (Matsson et al. 2009). Hoechst 33342 was added to the culture media at 0.5 μg/ml. Fluorescence imaging of Hoechst 33342 after cultivation for 0, 1, 3, 6 and 24 h was carried out by using a LEICA AM6000 equipped with LAS AF Lite analysis software (Leica Microsystems, Wetzlar, Germany). The fluorescence intensity of Hoechst 33342 at 455 nm was measured for 40 nuclei each composed of about 40–80 pixels per cell and the mean values (± SD) of total pixels in the 40 nuclei were calculated.

### Real-time PCR

Total RNAs were prepared from TtT/GF, Tpit/F1 and LβT2 by using ISOGEN (Nippon Gene, Toyama, Japan) and converted to cDNAs with PrimeScript Reverse Transcriptase (Takara Bio Inc., Otsu Japan). Quantitative real-time PCR was performed by using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) and an ABI PRISM 7500 Real Time PCR System (Applied Biosystems, Foster City, Calif., USA). The real-time PCR primers for mouse genes used were as follows: for *Abcb1a*, 5'-TCTGGACGAAGCAACATCAG-3' (forward primer; F) and 5'-ACCTTGCCGTTCTGAATCAC-3' (reverse primer; R); for *Abcb1b*, 5'-TCTGGACGAAGCAACATCAG-3' (F) and 5'-TCCTTGACTTTGCCGTTCTC-3' (R); for *Abcc1*, 5'-

TTGCTCATCGGCTTAACACC-3' (F) and 5'-ATCCTTGGC CATGCTGTAGA-3' (R); for *Abcc4*, 5'-TTCAGCAACTGG GCAAGG-3' (F) and 5'-GCTGTCCATTGGAGGTGTTC-3' (R); for *Abcg2*, 5'-GCATTCCTCGATATGGCTTC-3' (F) and 5'-GACAGTTCGATGCCCTGATT-3' (R); for *Abcb10*, 5'-AGGATTGCAATAGCCAGAGC-3' (F) and 5'- ATGTGTCC CGTGTTCACAGA-3' (R); for TATA-box-binding protein (*Tbp*), 5'-GATCAAACCCAGAATTGTTCTCC-3' (F) and 5'-ATGTGGTCTTCCTGAATCCC-3' (R); and for *Sca1*, 5'-CCC CTACCCTGATGGAGTCT-3' (F) and 5'-GGCAGATGGGTA AGCAAAGA-3' (R). Fluorescent signals were normalized to that of *Tbp* and the threshold cycle (Ct) was set within the exponential phase of PCR. The relative gene expression was calculated by comparing the cycle times for each target PCR. Cycle threshold values were converted to relative gene expression levels by using the $2^{-(\Delta Ct\ sample-\Delta Ctcontrol)}$ method.

## cDNA microarray

cDNA microarrays were performed by making use of the custom analysis services of Kurabo Industries (Osaka, Japan) and by using qualified total RNA samples from TtT/GF cells and the rat pituitary at postnatal day 60 as described in a previous paper (Cai et al. 2012).

## Animal experiments and immunohistochemical analyses of ABCG2

All animal experiments were approved by the Committee on Animal Experiments of the School of Agriculture, Meiji University. S100β-GFP transgenic rats, which were generated by fusing the S100β-promoter to the reporter gene green fluorescent protein (GFP; Itakura et al. 2007), were used to monitor the S100β-expressing folliculo-stellate cells. Male pituitaries of postnatal day 60 (P60) rats were surgically removed and fixed with HOPE Fixative System solution I (Polysciences, Warrington, Pa., USA) for 24 h at 4 °C, dehydrated with an ice-cold 1:1 solution of HOPE solution II and acetone for 2 h and three times with ice-cold acetone for 2 h, transferred immediately into prewarmed low-melting paraffin and incubated overnight at 55 °C. Embedded samples were sectioned at a thickness of 6 μm and mounted on glass slides (Matsunami, Osaka, Japan). After deparaffinization and hydration, sections were activated by an Immunosaver (Nisshin EM, Tokyo, Japan) in 0.05 % citraconic anhydride solution, pH 7.4, for 5 min at 115 °C and washed three times in 20 mM HEPES (pH 7.5) containing 100 mM NaCl, followed by treatment with blocking buffer composed of 0.4 % Triton-X100 and 10 % FBS in HEPES buffer, pH 7.5, for 60 min at room temperature. Then, sections were incubated for 16 h at room temperature with mouse monoclonal anti-human ABCG2 (1:200 dilution; Abcam, Cambridge, UK) or chicken IgY anti-jellyfish GFP (1:500 dilution; Aves Labs, Tigard, Ore., USA) as the primary antibody in the blocking buffer. After sections had been washed three times with HEPES buffer (pH 7.5) for 15 min, they were incubated for 2 h at room temperature with Cy3- or Cy5-conjugated donkey anti-mouse or anti-chicken IgY antibody (1:500 dilution) as the secondary antibody (Jackson ImmunoResearch, West Grove, Pa., USA) in the blocking buffer. Finally, sections were mounted in a Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, Calif., USA) and immunofluorescence was observed with a BZ-8000 fluorescence microscope (Keyence, Osaka, Japan).

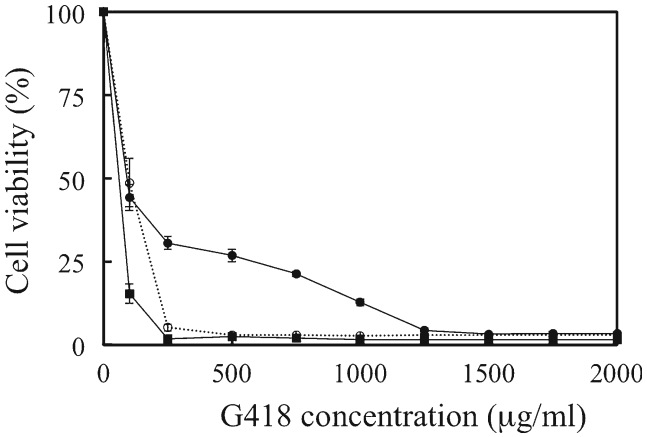


**Fig. 1** Viability of pituitary-derived cell lines after culture with the antibiotic G-418. Cell viability was examined by addition of G418 to give a final concentration as indicated on the *horizontal axis* to pituitary-tumor-derived cell lines: TtT/GF (*closed circles*), Tpit/F1 (*open circles*) and LβT2 (*closed squares*) cells

## Statistical analyses

The fluorescence intensity of Hoechst 33342 was statistically examined by the Bartlett test to confirm the data distribution, followed by one-way analysis of variance with Dunnett's post hoc test, or by the Kruskal-Wallis test, followed by the Mann–

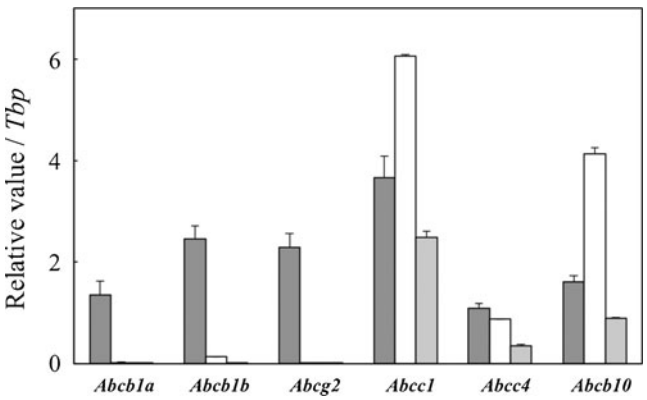


**Fig. 2** Real-time polymerase chain reaction (PCR) analysis of the expression level of ATP-binding cassette (ABC) transporter genes. Expression levels of ABC transporter genes were quantified by real-time PCR. The amounts are presented as values relative to those of the TATA-box-binding protein gene (*Tbp*) for TtT/GF (*dark gray bars*), Tpit/F1 (*open bars*) and LβT2 (*light gray bars*) cells

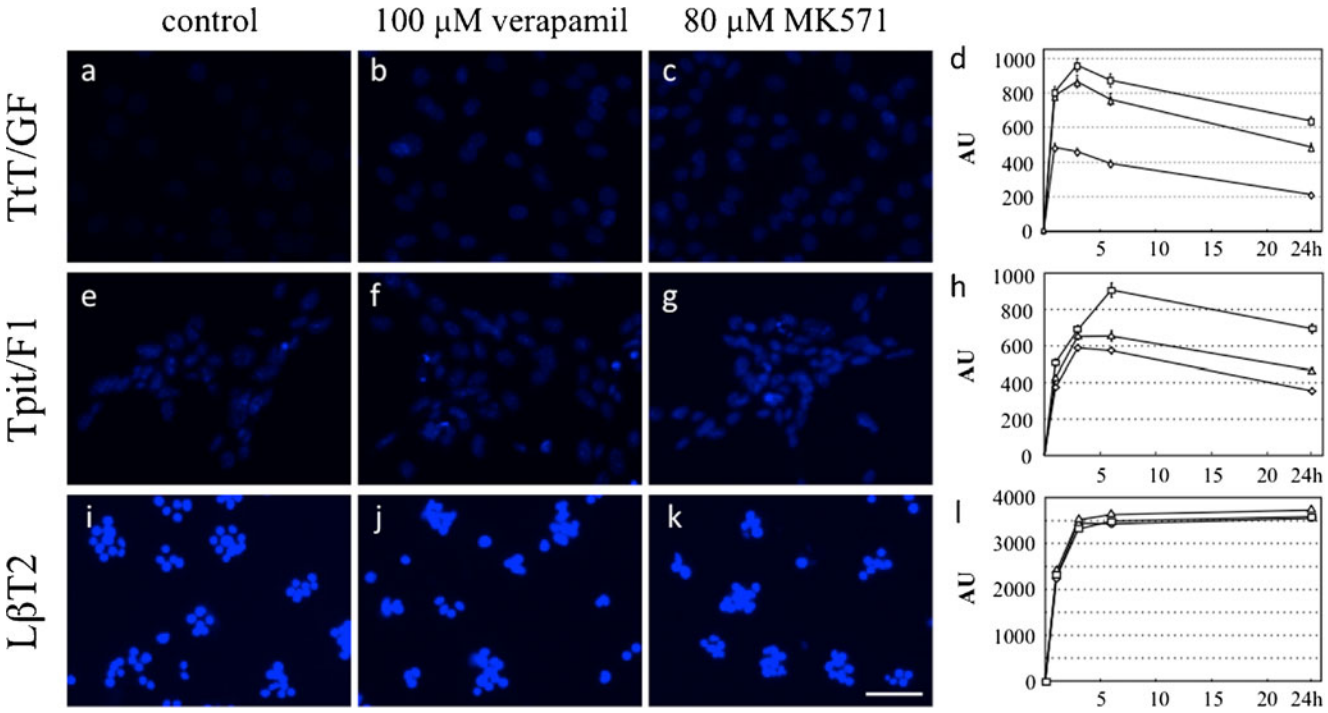


**Fig. 3** Fluorescence images after treatment with inhibitors, verapamil and MK571. Fluorescence images after 24-h treatment with verapamil at 100 μM (**b**, **f**, **j**) and MK571 at 80 μM (**c**, **g**, **k**) are shown in comparison with the controls (**a**, **e**, **i**). Time course of fluorescence intensity (*AU* arbitrary units) of Hoechst 33342 in the nuclei ($n$=40), measured as mean intensity of 40 nuclei each composed of 40–80 pixels per cell, increased after the start of cultivation in the absence (*diamonds*) and presence (*triangles*) of verapamil and the presence (*squares*) of MK571. Data of the control, verapamil at 100 μM and MK571 at 80 μM are shown in **d**, **h**, **l**

Whitney *U*-test. Differences were considered statistically significant at $P<0.05$.

## Results

### Cell viability after culture with G418

Cell viability after culture with the antibiotic G418 was examined for the TtT/GF, Tpit/F1 and LβT2 cells (Fig. 1). The survival rate of the Tpit/F1 and LβT2 cells was lower than 10 % at 250 μg/ml, which can be considered a "normal" level for this treatment. In contrast, TtT/GF cells still showed 30 % viability at around 250 μg/ml; viability of less than 10 % was obtained eventually at 1,250 μg/ml. The strong resistance to the antibiotic G418 indicates that TtT/GF cells have a drug efflux ability, possibly attributable to the participation of some type(s) of ABC transporters.

### Real-time PCR of ABC transporters

To confirm the expression levels of the transporter genes, real-time PCR was performed for *Abcb1a*, *Abcb1b*, *Abcc1*, *Abcc4* and *Abcg2* (efflux transporters) and for *Abcb10* (non-efflux transporter) by using cDNAs prepared from TtT/GF, Tpit/F1 and LβT2 cells. The values assayed were calculated as relative amounts against that of *Tbp*. As shown in Fig. 2, *Abcb1a*, *Abcb1b* and *Abcg2* were expressed in the TtT/GF cells, whereas a tiny amount of *Abcb1b* was observed in the Tpit/F1 cells. The

**Table 1** Effect of inhibitors on Hoechst 33342 efflux in the three studied cell lines (*TtT/GF*, *Tpit/F1*, *LβT2*). Fluorescence intensity of Hoechst 33342 was measured after cultivation for 24 h and tabulated. Mean intensity (± SD) of 40 nuclei, each composed of 40–80 pixels per cell, is indicated. Significant differences were found between the control and inhibitors (*$P$<0.05; **$P$<0.01)

| Treatment | Concentration (μM) | TtT/GF | Tpit/F1 | LβT2 |
|---|---|---|---|---|
| Control | - | 214.3±11.8 | 355.0±16.8 | 3547.5±51.9 |
| Verapamil | 50 | 464.9±29.3** | 460.7±23.8** | 3677.7±31.3 |
| | 100 | 488.9±20.0** | 467.4±15.0** | 3727.9±24.8* |
| MK571 | 40 | 392.8±19.2** | 525.1±23.1** | 3600.8±30.4 |
| | 80 | 638.6±23.9** | 694.7±25.6** | 3584.1±31.2 |

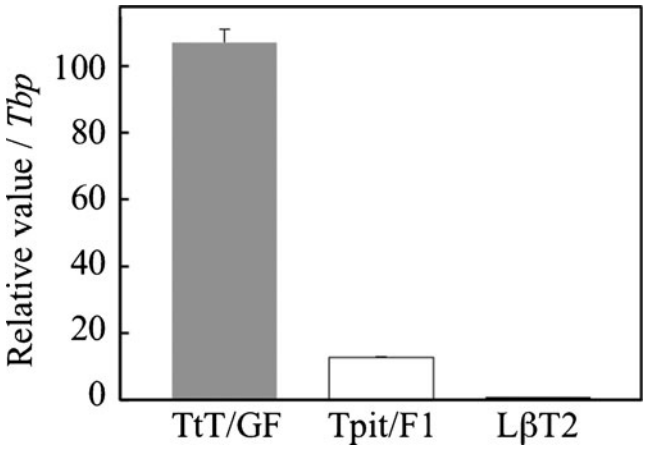


**Fig. 4** Real-time PCR analysis of the expression level of *Sca1*. Expression level of *Sca1* was quantified by real-time PCR. The amounts are presented as values relative to those of the TATA-box-binding protein gene (*Tbp*) for TtT/GF, Tpit/F1 and LβT2 cells

other transporters, namely *Abcc1*, *Abcc4* and *Abcb10*, were commonly present in the three cells.

## Inhibition of Hoechst 33342 efflux by using inhibitors for ABC transporters

Efflux of Hoechst 33342 was examined in the absence or presence of verapamil and MK571, inhibitors of the ABC transporter. Fluorescent microscopy images showed an apparent increase in Hoechst 33342 in TtT/GF and Tpit/F1 cells with 100 μM verapamil and 80 μM MK571 (Fig. 3). Fluorescence intensities reached an equilibrium by 6 h after addition of Hoechst 33342 and declined slightly by 24 h (Fig. 3d, h, l), except in LβT2 cells. Densitometry of the fluorescence imaging at 24 h was carried out for 40 nuclei. As listed in Table 1, the intensity significantly increased by about 2.3-fold (100 μM verapamil) and 3.0-fold (80 μM MK571) in the TtT/GF cells and by about 1.3-fold (100 μM verapamil) and 2.0-fold (80 μM MK571) in the Tpit/F1 cells, whereas no apparent change occurred in the LβT2 cells.

## Real-time PCR of *Sca1*

Cells expressing *Abcb1a*, *Abcb1b* and *Abcg2* are considered to have the properties of stem/progenitor cells. Since *Sca1* is

**Table 2** List of expressing genes ranking in the top 20 transcripts in the TtT/GF cells. Microarray analysis of expressing genes was performed in the TtT/GF cells and in the postnatal rat anterior lobe at 60 days old (*ND* not detected). The top 20 most abundant transcripts of the TtT/GF cells are listed with the signal value related to that of TATA-box-binding protein (*Tbp*), excluding genes of ribosomal and related proteins, in comparison with that of the anterior lobe of the pituitary gland (*Pituitary*)

| Rank | Gene name | Gene symbol | Ratio per Tbp | | Accession number |
|---|---|---|---|---|---|
| | | | TtT/GF | Pituitary | |
| 1 | Lectin galactose binding soluble 1 | Lgals1 | 101 | 1.0 | NM_008495 |
| 2 | Ferritin heavy chain 1 | Fth1 | 86 | 19.0 | NM_010239 |
| 3 | S100 calcium binding protein A4 | S100a4 | 85 | 0.2 | NM_011311 |
| 4 | Eukaryotic translation elongation factor 1 alpha 1 | Eef1a1 | 84 | 3.1 | NM_010106 |
| 5 | Actin beta, cytoplasmic | Actb | 83 | 17.6 | NM_007393 |
| 6 | Actin gamma, cytoplasmic 1 | Actg1 | 82 | 18.2 | NM_009609 |
| 7 | Vimentin | Vim | 79 | 3.7 | NM_011701 |
| 8 | Tumor protein translationally-controlled 1 | Tpt1 | 78 | 33.0 | NM_009429 |
| 9 | Procollagen type I, alpha 2 | Col1a2 | 76 | 1.1 | NM_007743 |
| 10 | Thymosin beta 4 | Tmsb4 | 73 | ND | NM_021278 |
| 11 | Secreted acidic cysteine-rich glycoprotein | Sparc | 72 | 4.1 | NM_009242 |
| 12 | Annexin A1 | Anxa1 | 70 | 0.3 | NM_010730 |
| 13 | Peptidylprolyl isomerase A | Ppia | 69 | 21.6 | NM_008907 |
| 14 | Secreted phosphoprotein 1 | Spp1 | 68 | 3.6 | NM_009263 |
| 15 | Ubiquitin C | Ubc | 66 | 5.0 | NM_019639 |
| 16 | Thymosin beta 10 | Tmsb10 | 66 | 3.4 | NM_001039392 |
| 17 | Procollagen type III, alpha 1 | Col3a1 | 64 | 1.1 | NM_009930 |
| 18 | Cathepsin B | Ctsb | 61 | 9.5 | NM_007798 |
| 19 | Selenoprotein P, plasma, 1 | Sepp1 | 60 | 4.6 | NM_001042613 |
| 20 | Aldo-keto reductase family 1 member A4 | Akr1a4 | 60 | ND | NM_021473 |

**Table 3** List of expressing genes characteristic in the TtT/GF cells (*ND* not detected). The signal value related to that of TATA-box-binding protein (*Tbp*) is listed in comparison with that of the anterior lobe of the pituitary gland at 60 days old (*Pituitary*)

| Gene name | Gene symbol | Ratio per Tbp | | Accession number |
|---|---|---|---|---|
| | | TtT/GF | Pituitary | |
| Stem/progenitor cells | | | | |
| SRY-box containing gene 2 | Sox2 | 0.6 | 0.4 | NM_011443 |
| SRY-box containing gene 9 | Sox9 | 0.8 | 0.0 | NM_011448 |
| CD44 antigen/GP90 lymphocyte homing | CD44 | 15.3 | 1.3 | NM_001039150 |
| Lymphocyte antigen 6 complex, locus A | Sca1/Ly6a | 55.2 | ND | NM_010738 |
| Nestin | Nes | 1.9 | 0.1 | NM_016701 |
| Bmi1 polycomb ring finger oncogene | Bmi1 | 6.7 | ND | NM_007552 |
| ATP-binding cassette,sub-family B, member 1A | Abcb1a | 0.8 | 0.0 | NM_011076 |
| ATP-binding cassette,sub-family B, member 1B | Abcb1b | 1.5 | 0.1 | NM_011075 |
| ATP-binding cassette,sub-family G, member 2 | Abcg2 | 1.4 | 0.2 | NM_011920 |
| Leucine rich repeat containing G protein coupled receptor 5 | Lgr5 | 0 | ND | NM_010195 |
| Early pituitary embryogenesis | | | | |
| Paired-like homeodomain transcription factor 1 | Pitx1 | 2.4 | 0.5 | NM_011097 |
| Paired-like homeodomain transcription factor 2 | Pitx2 | 2.1 | 4.1 | NM_001042502 |
| ISL1 transcription factor, LIM/homeodomain | Isl1 | 3.3 | 0.5 | NM_021459 |
| Sine oculis-related homeobox 1 homolog | Six1 | 0.9 | 1.5 | NM_009189 |
| Homeobox, msh-like 1 | Msx1 | 0.5 | 0.7 | NM_010835 |
| Homeobox gene expressed in ES cells | Hesx1 | 0 | ND | NM_010420 |
| Paired like homeodomain factor 1 | Prop1 | 0.1 | ND | NM_008936 |
| Paired box gene 6 | Pax6 | 0.4 | 0.5 | NM_013627 |
| Angiogenesis/endothelial cell | | | | |
| Endothelial-specific receptor tyrosine kinase/Tie2 | Tek | 0.1 | 0.6 | NM_013690 |
| Endoglin | Eng | 1.9 | 0.2 | NM_007932 |
| CD34/hematopoietic progenitor cell antigen | CD34 | 32.2 | ND | NM_133654 |
| Cadherin 5/VE-cadherin | Cdh5 | 0.1 | 1.7 | NM_009868 |
| Nitric oxide synthase 3, endothelial cell | Nos3 | 0.2 | 0.0 | NM_008713 |
| CD14 antigen | Cd14 | 1.4 | 0.3 | NM_009841 |
| Selectin, platelet | Selp | 2.2 | 0.0 | NM_011347 |
| Vascular cell adhesion molecule 1 | Vcam1 | 6.5 | 0.5 | NM_011693 |
| FMS-like tyrosine kinase 4/VEGFR3 | FLT4 | 0.1 | 0.1 | NM_008029 |
| Podoplanin | Pdpn | 7.1 | 0.7 | NM_010329 |
| Actin, alpha 2, smooth muscle, aorta | Acta2 | 33.9 | 0.2 | NM_007392 |
| Fibronectin 1 | FN1 | 58.2 | 0.2 | NM_010233 |
| Other | | | | |
| Collagen, type I, alpha 1 | Col1a1 | 25.1 | 0.2 | NM_007742 |
| S100 protein, beta polypeptide, neural | S100b | 5.7 | 2.0 | NM_009115 |

a known marker of stem/progenitor cells, real-time PCR was performed to measure the expression level of *Sca1*. As shown in Fig. 4, expression of *Sca1* was marked in TtT/GF cells, with the level being 116.3-fold that of *Tbp*. Tpit/F1 also expressed *Sca1* at a low level, i.e., at 15.57-fold that of *Tbp*, whereas LβT2 did not.

## Abundantly expressing genes in TtT/GF cells

To characterize TtT/GF cells further, we took the top 20 most abundantly expressing genes from the microarray data for the TtT/GF cells. As listed in Table 2, genes related to the pituitary

hormones were absent and many unique genes, such as *Lgals1*, *S100a4*, *Vim*, *Tmsb4x* and *Anex1*, were distinguished, the values of which were extremely low in the postnatal rat anterior lobe of the pituitary gland at 60 days old. *Vim* is especially characteristic, since it is known as a marker of mesenchymal cells, as is *Sca1* and if it is an originally expressing gene in the parent cell, this indicates that the TtT/GF cell has an extrapituitary origin. In Table 3, genes are listed that are involved in the stem/progenitor, early pituitary organogenesis and angiogenesis/endothelial cells. *CD44*, *Sca1*, *CD34*, *Acta2* and *FN1* have relatively high levels compared with *Tbp*, whereas genes related to early pituitary embryogenesis have a low level and *S100b* is 6.5-fold that of *Tbp*. All genes described above exhibit low activity in the postnatal rat anterior lobe at 60 days old. The gene expression profiles in Tables 2 and 3 indicate that the TtT/GF cell is of extrapituitary origin.

### Localization of ABCG2 in folliculo-stellate cells

Cell lines are frequently posulated to express a slightly different set of genes from that of the parent cells. To eliminate this possibility, the localization of ABCG2 in the pituitary folliculo-stellate cells was examined by using an S100β-GFP transgenic rat (Itakura et al. 2007), which is a suitable model animal for monitoring of S100β-expressing folliculo-stellate cells with S100β promoter-dependent GFP expression. Fluorescence of GFP was quenched by fixation and then double immunostaining of ABCG2 and GFP was performed. As shown in Fig. 5, the presence of cells positive for ABCG2 and GFP was observed in addition to cells positive only for one protein (arrowhead), indicating that cells positive for each protein are not homogeneous populations.

## Discussion

We recently demonstrated that the TtT/GF cell line expresses the paired-related homeobox transcription factors, *Prrx1* and *Prrx2* (Higuchi et al. 2013; Susa et al. 2012), which are known to play crucial roles in the development of the mesenchymal tissues. The present study has further revealed that TtT/GF cells have the ability to resist the antibiotic G418, to efflux Hoechst 33342 and to uniquely express *Abcg2* and *Sca1*, in addition to other characteristic genes. Identification of these molecules might elucidate novel aspects for a better understanding of the biological role of TtT/GF cells.

The TtT/GF cell line was initially established from a pituitary tumor line induced by radiothyroidectomy and is characterized by the presence of many lysosomes and intermediate filaments in the cytoplasm, phagocytic activity, follicle formation and the expression of GFAP and S100 genes, which have properties similar to those of the folliculo-stellate cells in the pituitary (Inoue et al. 1992). Subsequently, various characteristics and functions of TtT/GF cells have been reported as follows: TtT/GF produces interleukin-6 (IL-6, an autocrine growth factor; Renner et al. 1997), vascular endothelial growth factor (VEGF, a highly potent angiogenic factor; Lohrer et al. 2001), annexin 1 (a mediator of early delayed glucocorticoid feedback action; Traverso et al. 1999), cytokine-induced neutrophil chemoattractant (CINC; Zhang et al. 1997), outward $K^+$ channels (similar to those of the $K^+$ channels in glial and Schwann cells; Yamasaki et al. 1997), leptin (a circulating hormone secreted mainly by adipose tissue; Jin et al. 2000), ciliary neuronotrophic factor (CNTF, stimulation of growth hormone and prolactin production; Perez Castro et al. 2000), IL-11 (Perez Castro et al. 2000) and PTTG1 (pituitary tumor transforming gene-1; Vlotides et al. 2006). Taking this accumulated knowledge together with the present results, the TtT/GF cell may have the property of differentiation plasticity.

The newly identified molecules in the present study, namely *Abcb1a*, *Abcb1b*, *Abcg2* and *Sca1*, might provide us with novel clues to resolve the function of the TtT/GF cells, since these molecules are associated with markers of stem/progenitor cells. ABCB1 (also known as MDR1, P-glycoprotein and Pgy1), which is composed of ABCB1a and ABCB1b in the mouse, participates in the resistance to the antibiotic G418 (Theile et al. 2010) and is known to be present in the neural stem cell (Islam

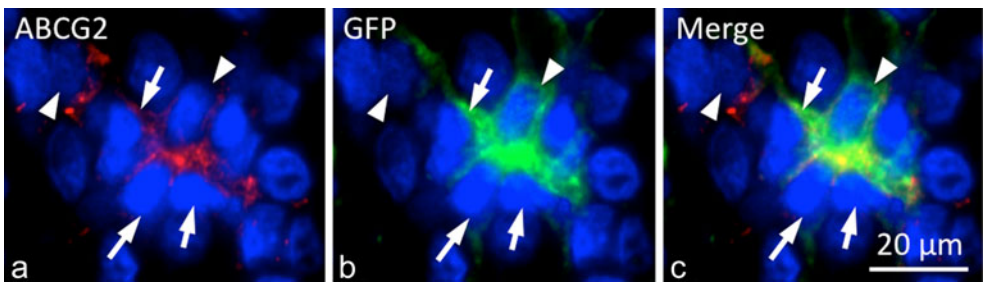


**Fig. 5** Immunohistochemistry of ABCG2 and GFP. To examine colocalization of ABCG2 and S100β, pituitary sections of S100β-GFP (green fluorescent protein) transgenic rats at 60 days old were taken, since the rat is a suitable model for monitoring of S100β-expressing folliculo-stellate cells with GFP. ABCG2 (**a**, *red*) and GFP (**b**, *green*) were detected with each antibody and the merged image is shown in **c** together with DAPI staining (*blue* nuclei, *arrows* cells positive for both proteins, *arrowheads* cells positive for only one of the proteins)

et al. 2005; Sawicki et al. 2006). ABCG2 (also termed BCRP and MXR) was first identified in a breast cancer cell line (Doyle et al. 1998) and is known to be a determinant of the Hoechst-negative phenotype of the side population (SP) cells found in a wide variety of stem cells (Bunting 2002; Herman et al. 2012; Sarkadi et al. 2004). Thus, the existence of ABCB1a, ABCB1b and ABCG2 in the TtT/GF cells indicates that this cell line has some characteristics of stem/progenitor cells. Additionally, we have confirmed the existence of another stem cell marker, Sca1 (also called Ly-6A/E), first found on the surface of several murine marrow stem cell subtypes (Spangrude et al. 1988) and later identified in SP cells (Goodell et al. 1996; Gussoni et al. 1999). Notably, Jiang et al. (2002) have found *Abcg2*-expressing cells in the mesenchymal stem cell fraction. We have observed that Tpit/F1 also expresses *Sca1* at a low but consistent level in comparison with that of TtT/GF, probably indicating the stemness of this cell line. Indeed, this cell line is capable of transformation into skeletal muscle cells (Mogi et al. 2004).

The characteristics described above give rise to question about the origin of the TtT/GF cell. By reference to the efflux of Hoechst 33342, Vankelecom's group separated SP cells into two sub-fractions of cells, Sca1$^{high}$ (composing 60 % of the SP) and non-Sca1$^{high}$, based on the content of Sca1 (Chen et al. 2009; Vankelecom 2010). They further found, by microarray analyses of the two subsets, that the Sca1$^{high}$ fraction showed a gene expression profile similar to that of endothelial cells with a higher expression of *Oct4*, *Nes* and *Bmi1*, whereas the non-Sca1$^{high}$ revealed a high expression of *Sox2* together with that of *Hesx1*, *Lhx4*, *Prop1*, *Pax6*, *Otx2* and genes of specific notch pathway components considered to be markers of pituitary stem/progenitor cells. Accordingly, two different cell lineages, both of which show properties of stem/progenitor cells, are present in the anterior lobe of the pituitary gland. Meanwhile, a number of characteristic genes described in this study, such as *Abcg2* (Elkind et al. 2005), *Sca1* (Batts et al. 2011; Ishikawa et al. 2006) and *Vim* (Dutsch-Wicherek 2010), are known to be expressed in tumor cells. Moreover, a cell line is known to express a slightly different set of genes from its parent cells, as mentioned above. The TtT/GF cell line, indeed, has several characteristics similar to folliculo-stellate cells of the pituitary but careful attention is required to interpret the data. At least in some of the pituitary folliculo-stellate cells, we have confirmed the existence of ABCG2 by immunohistochemistry.

As can be seen by referring to Tables 2 and 3, genes characteristic of pituitary gland transcription factors Pitx1 and Pitx2 are expressed at a level similar to that of ABC transporters ABCB1a, ABCB1b and ABCG2. On the other hand, TtT/GF cells also express genes characteristic of mesenchymal cells (namely *Vim*, *Col*Iα*2* and *CD44*) and vascular/endothelial cells (namely *CD34*, *Vcam1*, *Acta2* and *FN1*), which are not relevant to cells originating from the epithelial oral ectoderm. Thus, whether the TtT/GF cell is derived from an extrapituitary origin is of interest. Recently, epithelial-mesenchymal transition (EMT), in which cells lose their epithelial characteristics and acquire more migratory mesenchymal properties, has been frequently reported (Lee et al. 2006; Taube et al. 2010). Indeed, Table 2 shows many genes associated with EMT, including *Fth1* (Zhang et al. 2009), *S100a4* (Strutz et al. 1995), *Vim* (Korsching et al. 2005), *Tmsb4* (Huang et al. 2007) and *Anxa1* (Maschler et al. 2010), possibly reflecting the lineage of the TtT/GF cell.

In conclusion, we have demonstrated characteristic aspects of the non-endocrine cell line TtT/GF, such as high anti-drug resistance, Hoechst efflux ability, the expression of many stem cell markers and genes associated with mesenchymal and vasculogenesis/endothelium cells and EMT. TtT/GF cells might still be in an incomplete state in differentiation or transition. An active approach to advancing cellular transformation of the TtT/GF cell in the near future will provide us with valuable cell types more similar to a component cell of the pituitary.

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