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Characterization of Possible Tumor Stem Cells from Human Pituitary Adenomas

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ABSTRACT

Recent studies have reported the presence of stem/progenitor-like cells in human pituitary adenomas. Thus, the present study aimed to identify the tumor stem cells and find a marker that could be used to isolate these cells. First, we used the sphere-forming assay to isolate stem-like cells from surgically removed samples. Then, we examined the expression profiles of stem cell-associated factors and pituitary hormones in spheres grown under 2 culture conditions. Cells sampled from silent adrenocorticotrophic hormone-producing adenoma formed spheres expressing stem cell-associated factors ; however, these factors decreased as the cells differentiated. Depending on the culture conditions, sphere-forming cells could differentiate into hormone-producing cells from other lineages. Next, we used the established brain tumor stem-cell marker, i.e., CD133 (prominin 1), to isolate stem-like cells and investigate whether CD133 expression was correlated with the expression of stem cell-associated factors. The $CD133^+$ cells from 10 nonfunctioning adenomas upregulated the expression of stem cell-associated factors *SOX2* (SRY-box 2) and *OCT3/4* (organic cation transporter 3/4) more than did the $CD133^-$ cells ($P < 0.05$). Cumulatively, these results indicate the presence of possible tumor stem cells in human pituitary adenomas that can self-renew and differentiate into other hormone-producing cells. This study also shows that the $CD133^+$ phenotype can be used to identify tumor stem-like cells in human pituitary adenomas. (Jikeikai Med J 2016 ; 63 : 15-25)

Key words : silent adrenocorticotrophic hormone-producing adenoma, pituitary neoplasms, neoplastic stem cells, CD133

INTRODUCTION

Pituitary adenomas are a common benign intracranial tumor. The tumorigenesis has been considered to occur from mature pituitary cells in differentiation and development along 1 of the 3 lineages : (1) the adrenocorticotrophic hormone (ACTH)/pro-opiomelanocortin (POMC) lineage, (2) the growth hormone (GH)/prolactin (PRL)/thyroid-stimulation hormone (TSH) lineage, and (3) the follicular stimulating hormone (FSH)/luteinizing hormone (LH) lineage. Pituitary adenomas may excrete excess hormones, leading to particular manifestations, such as Cushing's disease and acromegaly. The first choice of treatment is transsphenoidal surgery, which can also be combined

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with chemotherapy and radiotherapy. However, some cases are difficult to manage because of resistance to treatment, rapid growth, and a high rate of recurrence.

Solid tumors consist of heterogeneous cells. Tumor tissues contain a subset of tumor stem cells, or cancer stem cells, which are pluripotent and can self-renew similar to normal stem cells. The tumor stem cells are thought to be correlated with tumor initiation, rapid and invasive growth in some tumors and cancers, and high resistance to therapies[1-3]. Thus, the identification and characterization of tumor stem cells is of great importance from both pathogenic and therapeutic perspectives.

Two methods are commonly used to identify and characterize tumor stem cells. One is the sphere-forming assay method, which is frequently used to isolate clonally proliferating stem cells as floating cell clusters ("spheres") by culturing them in a unique, nonserum medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). This method is generally used to identify the self-renewal properties of stem cells[4]. The other method uses markers, such as tumor-specific transcription factors and cell surface proteins, to identify and isolate tumor stem cells. Stem-like cells are present in human pituitary adenomas in vitro[5-7]. Moreover, unclassifiable clinical cases of pituitary adenomas, expressing multiple hormones beyond the pituitary lineage, suggest that they are derived from stem cells instead of mature pituitary cells[8]. However, few reports have described the detailed characterization of tumor stem cells from human pituitary adenomas.

Therefore, in the present study, we isolated tumor stem cells from human pituitary adenomas using the sphere-forming assay method. Next, we characterized the expression profiles of stem cell-associated factors and pituitary hormones in these cells and examined their ability to undergo stem cell-like differentiation (differentiation pluripotency) by changing the culture condition. Finally, we isolated cells positive for CD133, a marker of brain tumor stem cells, from the human pituitary adenomas and examined the expression of stem cell-associated factors in these cells.

Materials and Methods

This study was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from the subjects before they underwent surgery, and this study was approved by the ethics committees of both Toranomon Hospital and the Tokai University School of Medicine.

1. Tissue samples

The tissue samples of pituitary adenomas diagnosed as nonfunctioning adenomas were obtained from 11 patients who underwent transsphenoidal surgery at Toranomon Hospital. One of these samples, from a tumor diagnosed as a silent ACTH-producing adenoma, was used for a sphere-forming assay experiment. Although this adenoma was a clinically nonfunctioning tumor, its tumor tissue was confirmed with immunohistologic examination to express ACTH. Silent ACTH-producing adenomas are defined as pituitary adenomas that show positive staining for ACTH in immunohistological examinations but are not associated with clinical or biological signs of hypercortisolemia[9]. The other 10 samples were used for cell sorting and flow cytometry analysis (Table 1).

Immediately after surgery, the tumor tissues were collected and rinsed with phosphate-buffered saline (PBS), dissected, and dissociated into single cells by incubation in a cell detachment solution (Accutase®, Innovative Cell Technologies, Inc., San Diego, CA, USA) for 10 minutes at 37°C.

2. Sphere-forming and differentiation assays

Cell cultures

For the sphere-forming assay experiment, isolated tumor cells from silent ACTH-producing adenoma were seeded at a density of 2.5×10^5 cells/ml in 60-mm Petri dishes. The assay contained serum-free medium (NeuroCult® NCFC Serum-Free Medium, StemCell Technologies, Vancouver, BC, Canada), supplemented with commercial supplements (StemCell Technologies), 20 ng/ml EGF (Thermo Fisher Scientific, Waltham, MA, USA), 20 ng/ml bFGF (Thermo Fisher Scientific) and 200 U/ml penicillin/streptomycin (Thermo Fisher Scientific). Tumor cells were incubated at 37°C in 5% CO_2, and the medium was changed after 3 days. In the sphere-formation assay, the spheres were evaluated with immunohistochemical examination and gene expression analysis on day 7.

The differentiation assay investigates the ability of the sphere-forming cells to differentiate. For this assay, half of the spheres were harvested after the sphere-forming assay

Table 1. Characteristics of 11 clinically nonfunctioning adenomas

Case	Sex	Pathological Diagnosis	Age	Definitive Diagnosis
1	M	FSH-β (very weak)	48	Gonadotroph adenoma
2	F	Negative	45	Null cell adenoma
3	F	GH, 1%-2% ; TSH-β, 30-40%	44	Silent subtype 3 adenoma
4	M	TSH-β, 20% ; FSH-β, 20-30%	67	Silent TSH adenoma
5	M	FSH-β (very weak)	62	Gonadotroph adenoma
6	M	FSH-β (very weak)	63	Gonadotroph adenoma
7	M	FSH-β, 30%-40% ; LH-β (very weak)	69	Gonadotroph adenoma
8	M	FSH-β (very weak)	71	Gonadotroph adenoma
9	F	FSH-β, 30%-40% ; LH-β, 10-20%	67	Gonadotroph adenoma
10	F	Negative	60	Null cell adenoma
11	M	ACTH, 30%-40%	36	Silent ACTH adenoma

Cases 1 to 10 were used in FACS analysis. Case 11 was used in sphere-forming assay experiment. We chose these cases according to the clinical diagnosis. The definitive diagnosis, made according to World Health Organization classifications, was based on pathological findings from the surgery.

on day 7 and transferred to a differentiation medium in which 10% fetal bovine serum was added to the sphere-forming medium and incubated for 4 more days until day 11. The spheres were also examined for changes in immunohistochemical staining and gene expression.

Immunohistochemical staining

Immunohistochemical staining of pituitary hormones was performed on day 7 for spheres from the sphere-forming assay and on day 11 for spheres from the differentiation assay. Spheres from both treatments were collected and fixed with 4% paraformaldehyde for 30 minutes at 37°C. The spheres were then rinsed in PBS, permeabilized with Triton X-100 (0.3% in PBS), and blocked with 10% normal goat or normal rabbit serums for 60 minutes at room temperature.

The spheres were stained overnight with the following primary antibodies at room temperature : GH (rabbit polyclonal antibody, dilution 1 : 400 ; Dako, Glostrup, Denmark), PRL (rabbit polyclonal antibody, 1 : 400 ; Dako), ACTH (mouse monoclonal antibody, diluted 1 : 200 ; Dako), TSH (mouse monoclonal antibody, diluted 1 : 100 ; Advanced Immunochemical Inc., Long Beach, CA, USA), FSH (mouse monoclonal antibody, diluted 1 : 100 ; Beckman Coulter, Fullerton, CA, USA), LH-β (mouse monoclonal antibody, diluted 1 : 200 ; Beckman Coulter), and glycoprotein-hormone subunit α (αGSU) (rabbit polyclonal antibody, diluted 1 : 200 ; National Hormone and Pituitary Program/National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA). Primary antibodies were omitted for the negative control.

Then the spheres were incubated with Alexa 488-conjugated goat anti-mouse and anti-rabbit secondary antibodies for 60 minutes at room temperature. Nuclei were stained with TOTO-3 stain (Thermo Fisher Scientific) and mounted in 1,4-diazabicyclo[2.2.2]octane and then observed with confocal microscopy.

Real-time quantitative reverse transcriptase-polymerase chain reaction

Transcriptional factors, *SOX2* (SRY-box 2), *OCT3/4* (organic cation transporter 3/4), and *CD133* (prominin 1) *gene*, play a critical role in maintaining embryonic stem cells' undifferentiated state. Hence, the changes in mRNA expression of these factors were examined in both of the sphere-forming and differentiation assays. Because the number of harvested spheres was not enough for RT-PCR, each bar of Figure 5 denotes a single experiment using all spheres. Total RNA was extracted with RNeasy mini kits (QIAGEN, Valencia, CA, USA) and reverse-transcribed into complementary DNA with a QuantiTect Reverse Transcription Kit (QIAGEN). We assayed the messenger RNA (mRNA) of stem cell markers and stem cell-associated transcription factors, such as *SOX2*, *OCT3/4*, and *CD133* gene as well as mRNA of the pituitary development-associated factor *GATA2* (GATA binding protein 2) as a marker of differentiation. Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a reaction mixture containing SYBR Green fluorescent dye (DyNAmo SYBR Green qPCR Kit ; Finnzymes, Espoo, Fin-

Table 2. Primer sequences used in quantitative reverse transcriptase polymerase chain reaction analysis

Gene	Sequence (5′ to 3′)
ACTB	Forward : 5′-CCCAGCACAATGAAGATCAA-3′
	Reverse : 5′-ACATCTGCTGGAAGGTGGAC-3′
SOX2	Forward : 5′-CATCACCCACAGCAAATGAC-3′
	Reverse : 5′-AAGTTTTCTTGTCGGCATCG-3′
OCT3/4	Forward : 5′-TCAGGAGATATGCAAAGCAGAA-3′
	Reverse : 5′-CCTCTCACTCGGTTCTCGATAC-3′
CD133 gene	Forward : 5′-GCTGTAACAAATGTGGTGGAGA-3′
	Reverse : 5′-TGCCACAAAACCATAGAAGATG-3′
GATA2	Forward : 5′-AAGGCTCGTTCCTGTTCAGA-3′
	Reverse : 5′-TTCTGCCCATTCATCTTGTG-3′

land). Primers (Table 2) were designed with Primer3 software program (Whitehead Institute for Biomedical Research). Each RT-PCR reaction was run with Opticon Monitor 3 software (Bio-Rad Laboratories Inc., Hercules, CA, USA) and repeated in triplicate. The standard curve method was used to evaluate the relative expression levels of stem cell markers and transcription markers. *ACTB* (actin, beta) was used as the internal control and to normalize the relative expression levels to facilitate the comparison between the groups.

3. Cell sorting and flow cytometry analysis for CD133$^+$ cells in human pituitary adenomas

To investigate CD133$^+$ cells in human pituitary adenomas, 10 clinically nonfunctioning adenomas were examined in the flow cytometry analysis (Table 1).

Flow cytometry analysis was performed for each patient's specimen. The dispersed tumor cells were stained for 30 minutes at room temperature with mouse anti-human CD133/1 allophycocyanin-conjugated antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), mouse anti-human CD34 Phycoerythrin-conjugated antibody (Beckman Coulter), and mouse anti-human CD45 Pacific Blue-conjugated antibody (eBioscience, San Diego, CA, USA). Both CD34 and CD45 were used to eliminate hematopoietic cells, and only CD34$^-$/CD45$^-$ cells were considered as pituitary tumor cells. We focused on the CD133 cell surface protein because *CD133* gene was expressed more highly in undifferentiated spheres, suggesting that it is a potential marker of tumor stem cells in human pituitary adenomas. Living, labeled cells (negative for propidium iodide staining) were analyzed by means of flow cytometry with a FACS Aria cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

4. Statistical analysis

All data were analyzed by descriptive and inferential statistics (percentage, mean and *t*-test) using IBM SPSS Statistics (version 22.0 ; IBM Corp., Armonk, NY, USA). An unpaired 2-tailed Student's *t*-test was used to compare the means of parameters between the groups. A value of $P < 0.05$ was assumed significant for all tests.

Results

1. Immunohistochemical examination of the human pituitary adenoma

Immunohistochemical staining of pituitary hormones in the formalin-fixed paraffin-embedded (FFPE) section of this silent ACTH-producing adenoma pituitary adenoma was obtained from Toranomon Hospital (Fig. 1). Before the surgery, the adenoma was diagnosed as a nonfunctioning tumor ; however, the production of ACTH was confirmed with immunohistochemistry. The other pituitary hormones, including GH, αGSU, PRL, LH, FSH, and TSH, were found to be negative for staining in the sample tissues.

2. Development of spheres in sphere-forming and differentiation assays

After 3 days of culturing, sphere formation occurred at a rate of approximately 0.1% to 0.5% of spheres/cells seeded. Almost all other single cells were mortal. The spheres formed were smooth and rounded and, therefore, could be easily distinguished from cell aggregations (Fig. 2). We randomly collected 8 spheres and measured each of them on day 7 (sphere-forming assay) and day 11 (differentiation assay). On day 7 of the sphere-forming assay, the spheres had reached a diameter of 88.1 ± 12.1 μm (mean ± SD, $n = 8$). Once the spheres had been transferred to the differentiation assay, they expanded to 121.8 ± 19.8 μm (mean ± SD, $n = 8$) on day 11.

3. Immunohistochemical examination in sphere-forming and differentiation assays

On day 7 of the sphere-forming assay, the immunohistochemical examination revealed many ACTH$^+$ cells were founded in the sphere, as observed in the FFPE section of

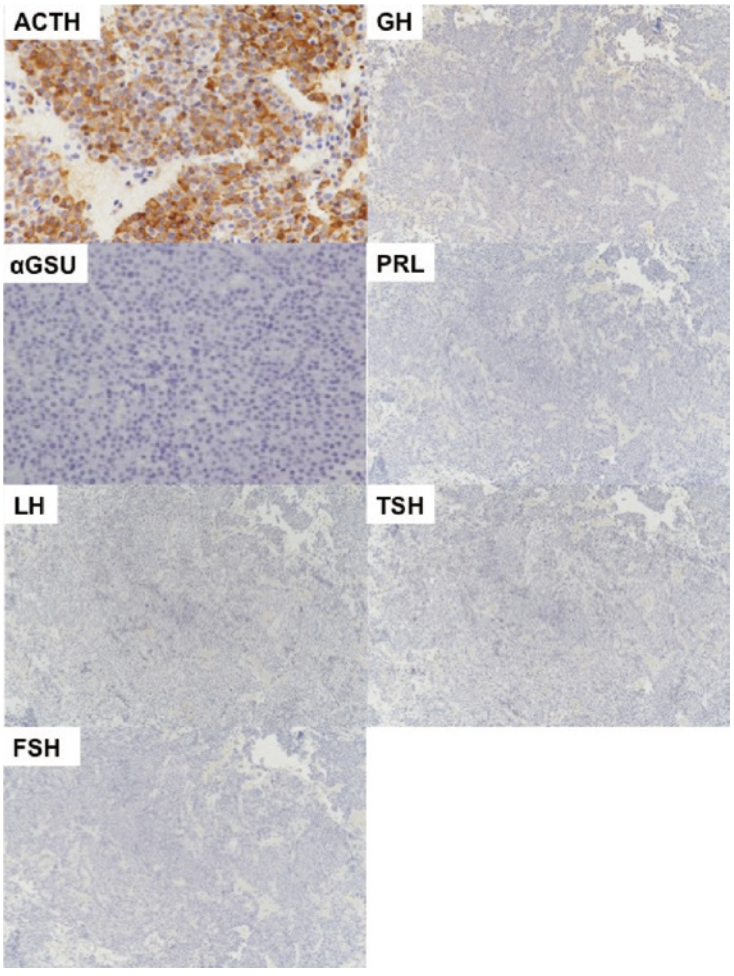

Fig. 1. Immunohistochemical staining of pituitary hormones in the formalin-fixed paraffin-embedded section of silent adrenocorticotrophic hormone (ACTH)-producing adenoma.
Tumor cells were only positive for ACTH.

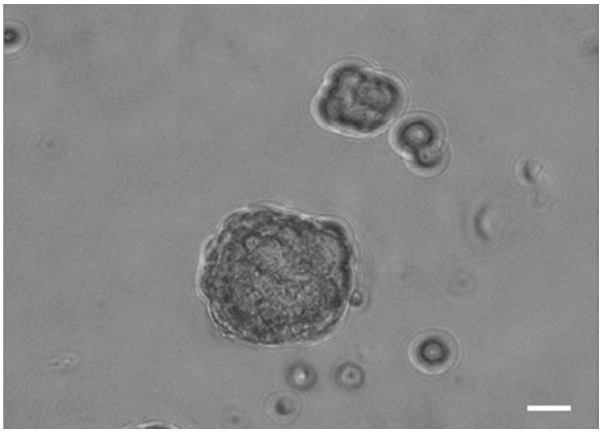

Fig. 2. Spheres from a clinically non-functioning pituitary adenoma.
Tumor stem-like cells proliferated in culture as floating cell clusters with a smooth and rounded form. Scale bar represents 20 μm.

the tumor (Figs. 1, 3A). In addition, LH and αGSU were detected in a few cells of the spheres ; however, these hormones could not be detected in the FFPE section of the original tumor (Figs. 1, 3B, C). Although GH and PRL were faintly stained only on the sphere's surface, they were considered negative because of the incomplete cellular staining pattern (Figs. 3E, F). No FSH or TSH could be detected in the spheres (Figs. 3D, G). Intriguingly, double-staining for αGSU and ACTH revealed an aberrant co-expression pattern in some $ACTH^+$ cells (Fig. 4).

On day 11 of the differentiation assay, the immunohistochemical examination showed that the numbers of αGSU^+ and LH^+ cells in the spheres were greater than on day 7 (Figs. 3B, C). The expression pattern of other hormones seemed to be unchanged over the differentiation assay.

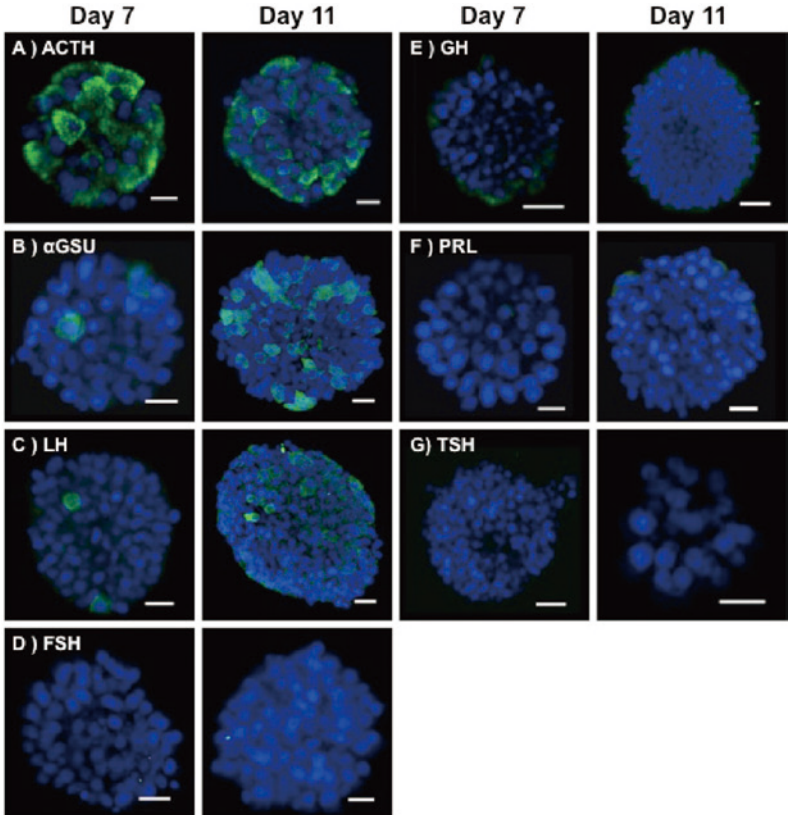

Fig. 3. Expression of pituitary hormones in 7- and 11-day-old spheres.
A) Adrenocorticotrophic hormone (ACTH) was highly expressed in spheres from silent ACTH-producing adenoma at both time points. B), C) Glycoprotein-hormone subunit α (αGSU) and luteinizing hormone (LH) were observed in a few sphere-forming cells on day 7. Eleven-day-old spheres had more αGSU$^+$ and LH-β$^+$ cells than the 7-day-old spheres. E, F) Spheres exhibited faint staining for GH and prolactin (PRL) in sphere's surface, but no cell specificity was observed. D, G) No follicular stimulating hormone (FSH) or thyroid-stimulation hormone (TSH) was detected. Scale bars represent 20 μm.

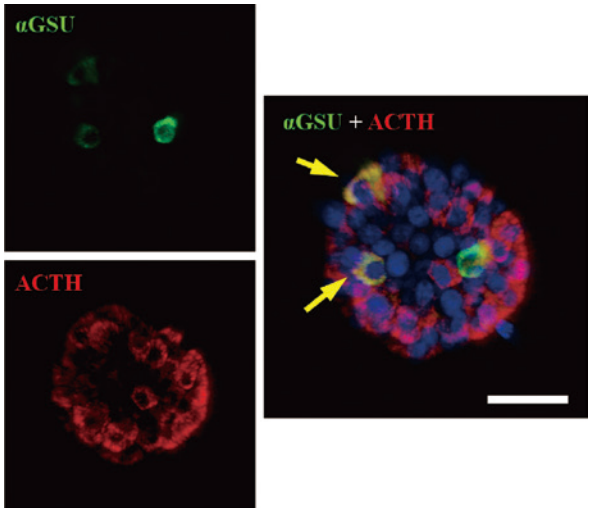

Fig. 4. Double staining for adrenocorticotrophic hormone (ACTH) and glycoprotein-hormone subunit α (αGSU) in sphere-forming cells on day 7.
An aberrant co-expression pattern occurred in some ACTH$^+$ cells (arrowheads). Scale bar represents 50 μm.

4. Gene expressions in sphere-forming and differentiation assays

The mRNA expression of *OCT3/4* and *SOX2* in the spheres on day 7 was suppressed in the spheres on day 11 of the differentiation assay (Figs. 5A, B). Additionally, the *CD133* gene expression was also suppressed in the spheres of day 11 compared to that of day 7 (Fig. 5C). Conversely, *GATA2*, a development-associated factor, was expressed at higher levels in the spheres of day 11 (Fig. 5D). These data suggest that 7-day-old spheres maintain a more undifferentiated state possessing tumor stem-like properties and become more differentiated by incubation in the differentiated medium as seen in 11-day-old spheres.

5. Cell sorting and flow cytometry analysis for CD133$^+$ cells in human pituitary adenomas

In another experiment, we investigated the presence of CD133$^+$ cells in human pituitary adenomas and charac-

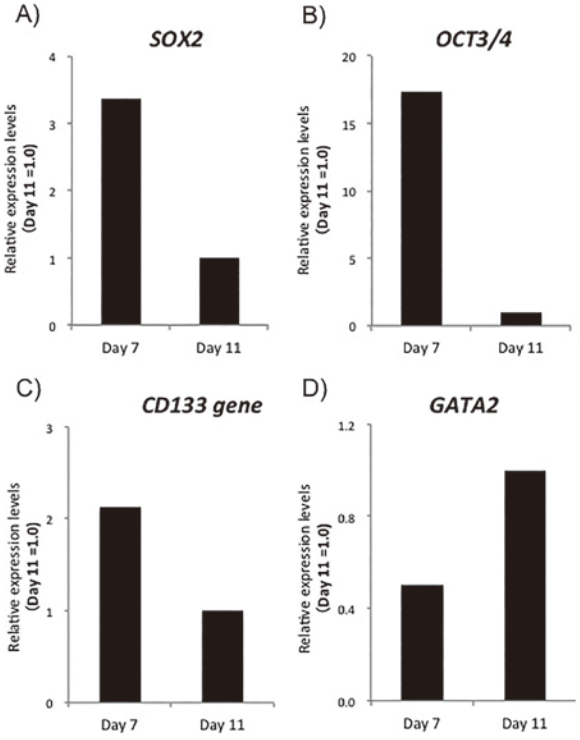

Fig. 5. Relative mRNA expression levels of stem cell-associated genes in 7- and 11-day-old spheres (day 11 = 1.0 as reference).
Stem-cell-associated markers, such as *OCT3/4*, *SOX2*, and *CD133* gene, were suppressed in the spheres on day 11 compared to that on day 7 ; however, the opposite trend was seen for *GATA2*, a factor associated with pituitary development. Each bar denotes 1 experiment.

terized gene expression in these cells. As expected, cell sorting and flow cytometry analysis with anti-human CD133 antibody indicated that only a small percentage of all pituitary tumor cells consist of $CD133^+$ (mean ± SD, 0.19% ± 0.12% ; $n = 10$) (Fig. 6A-D).

Populations of both $CD133^+$ cells ($CD133^+$, $CD34^-$, $CD45^-$) and $CD133^-$ cells ($CD133^-$, $CD34^-$, $CD45^-$) were assayed for the expression of genes associated with a stem cell phenotype, including *SOX2* and *OCT3/4*. Quantitative real-time RT-PCR revealed that the *SOX2* mRNA level was 5-times higher in $CD133^+$ cells than in $CD133^-$ cells. Likewise, *OCT3/4* expression was 8-fold higher in $CD133^+$ cells than in $CD133^-$ cells (Fig. 7). Both of these differences were statistically significant ($P < 0.05$). These data demonstrate that the expression levels of transcription factors critically involved in tumor stem cell-like properties are higher in cells expressing CD133.

Discussion

The cancer stem cell theory is supported by the presence of a small number of highly tumorigenic cells in tumors and cancers[1,2,10]. These tumor stem cells have been identified in an increasing variety of tumors, including those in the brain, prostate, colorectum, and breasts [11-21]. Because tumor stem cells are invasive and possess high drug-efflux ability, they are believed to be involved in tumor initiation and treatment resistance[22-24]. The identification of tumor stem cells can provide critical information for the treatment of different tumors. Previously, the presence of tumor stem cells in human pituitary adenomas, which are benign, hormone-secretary tumors composed of heterogeneous cells, has been controversial until the recent discovery of stem-like cells within them[5,25]. Hence, in the present study we aimed to identify and characterize tumor stem cells in human pituitary adenomas. The samples in this study were limited to nonfunctioning adenomas because we attempted to minimize how the variety of hormone-producing adenomas affected the results.

We considered tumor stem cells to simultaneously possess the ability to form floating cell clusters (spheres) and stem cell differentiation pluripotency. The former ability was evaluated in the sphere-forming assay by culturing dispersed cells in serum-free medium containing EGF and bFGF. Stem cell self-renew properties were evaluated in differentiation assays by investigating the production of pituitary hormones and the expression of stem cell-associated genes in the differential medium.

We found that the sphere-forming assay allowed us to isolate stem-like cells from a silent ACTH-producing adenoma. The pituitary tumor cells can survive for only a few days in primary culture ; however, in the sphere-forming assays, a small subset of tumor cells survived with sphere formation (Fig. 2). This survival suggests the ability of tumor cells to "self-renew," which is similar to that in stem cells observed in various tissues and tumors. Moreover, on day 7 of the sphere-forming assay, we observed the presence of αGSU^+ cells, the second earliest hormone following the ACTH expression in human pituitary development (Osamura RY, personal observation), which were not observed earlier in this tumor tissue (Figs. 1, 3B). Interestingly, αGSU was co-expressed with some $ACTH^+$ cells (Fig. 4), which indicate their "immaturity" and ability to "differ-

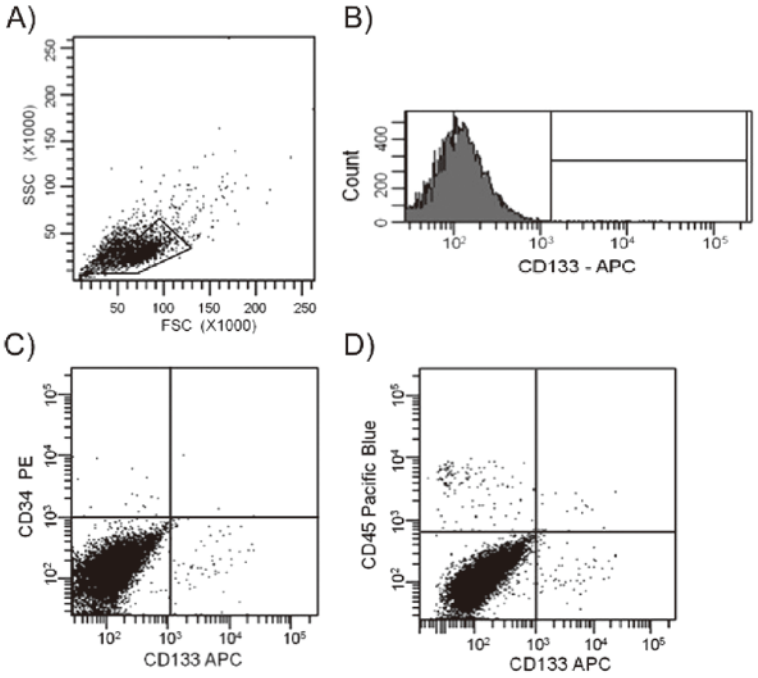

Fig. 6. Results from the FACS analysis of case 9.
A, B) CD133$^+$ cells of case 9 were 0.149%. Average number of CD133$^+$ cells in 10 cases was 0.19% ± 0.12%, (mean ± SD, n = 10). C, D) Tumor cells are divided into 2 populations: CD133$^+$ cells (CD133$^+$, CD34$^-$, CD45$^-$) and CD133$^-$ cells (CD133$^-$, CD34$^-$, CD45$^-$).

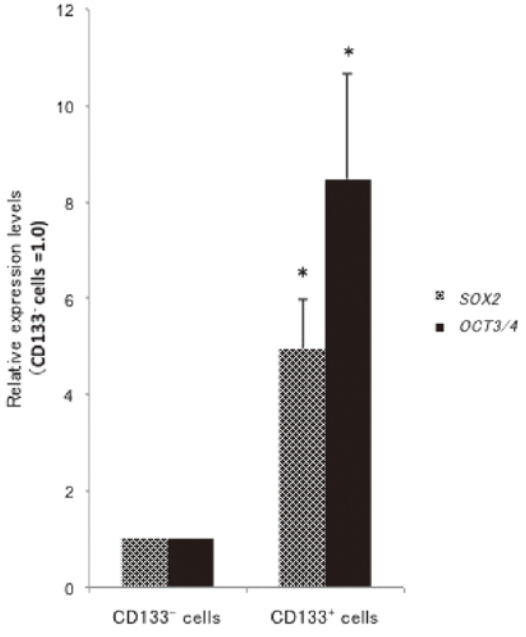

Fig. 7. Relative messenger RNA expression levels of *SOX2* and *OCT3/4* in CD133$^+$ and CD133$^-$ cells (CD133$^-$ cells = 1.0 as reference).
SOX2 messenger RNA expression was approximately 5 times higher in CD133$^+$ cells than in CD133$^-$ cells, and the *OCT3/4* expression was approximately 8 times higher. The bars represent mean ± SEM (n = 10 human pituitary adenomas). The asterisk (*) indicates $P < 0.05$.

entiate." Previous studies have found that silent ACTH-producing adenoma cells potentially possess certain features of gonadotroph cells, suggesting corticotroph and gonadotroph cells have a common stem/progenitor cell origin[26-28]. These stem/progenitor cells might be observed pathologically as both ACTH$^+$ and αGSU$^+$ cells in the early phase of sphere-forming cells. This possibility was also supported by the following differentiation assay, indicating that when the culture condition was changed, the sphere-forming cells started differentiating into the cells of other gonadotroph lineages, as documented by the more pronounced production of αGSU and LH (Figs. 3B, C).

We also found mRNA expressions of *SOX2*, *OCT3/4*, and *CD133* gene, which play a critical role in maintaining the undifferentiated state of embryonic stem cells[29,30], in the spheres of day 7, and the expression of these genes was suppressed with the exposure to the differentiation medium (Figs. 5A-C). In contrast, *GATA2*, which has been used as a marker of differentiation to gonadotroph or thyrotrophs cells[31,32], was found to be upregulated in the differentiation assay (Fig. 5D). In addition, *GATA2* is known to bind and transactivate the αGSU promoter and persist in an expression pattern coincident with αGSU, interacting with tran-

scription factor steroidogenic factor 1[33,34]. We showed that αGSU was detected more in the late phase than in the early phase of sphere-forming cells, which is considered to be related to high *GATA2* expression in the late phase. This reciprocal expression pattern, low expression of *SOX2*, *OCT3/4*, and *CD133* gene and high expression of *GATA2*, is considered to support the possibility that the sphere-forming cells can produce other pituitary hormones. As such, the expression profiles of stem cell-associated factors and pituitary hormones indicate that sphere-forming cells have stem-like properties and could be differentiated to some extent. On the basis of these results, we believe that tumor stem cells of human pituitary adenoma were successfully identified as sphere-forming cells in the present study.

In another experiment, we focused on CD133, a pentaspan membrane glycoprotein (transmembrane helix 5), as a marker for the identification and isolation of these cells from human pituitary adenomas. The $CD133^+$ cells have been isolated in a variety of normal human tissues and tumors and possess tumor stem-like characters, such as the ability to differentiate, proliferate, and form spherical clusters. These findings have led to CD133 being used as a tumor stem cell marker in brain tumors, colon cancer, hepatocarcinoma, and melanoma[12,13,18,19,35-38]. Previously, the expression of CD133 was also reported in human pituitary adenomas[39].

Therefore, the FACS analysis for $CD133^+$ cells was performed in the present study to identify tumor stem cells in human pituitary adenomas. As expected, we found that a minor proportion (0.19%) of pituitary adenoma cells were positive for CD133 (Fig. 6). Then, we compared the gene expression in $CD133^+$ cells and $CD133^-$ cells and found higher levels of *SOX2* and *OCT3/4* expression in $CD133^+$ cells than in $CD133^-$ cells (Fig. 7) ; this trend was also observed in the differentiation assay (Figs. 5A, B).

Although possible stem cell markers have previously been suggested by research in murine pituitary cells with stem-like characteristics[40-42], there have been few experimental reports on stem cell markers in human pituitary adenomas[6,7]. Thus, we believe the present study is of interest because it has identified CD133 as a marker for indicating tumor stem-like property in human pituitary adenomas.

However, we stimulated differentiation of sphere-forming cells in only a limited set of hormone-producing cells. For example, sphere-forming cells from silent ACTH-producing adenomas could not differentiate into the GH/PRL/TSH lineage regulated by pituitary-specific transcription factor 1. Thus, we could not completely demonstrate the differentiation pluripotency seen in stem cells. This lack of demonstration may have occurred because these cells, like adult stem cells, have a limited ability to differentiate from the aspect of cell lineage. This explanation seems logical given that human pituitary adenomas are known to be intensively affected by a combination of transcription factors, co-factors, and intracellular signal pathways. The stem/progenitor-like cells used in this study might have already been committed to the developmental pathways, leading them to become specific hormone-producing cell types. Also possible is that culture conditions or duration used in this study might not be adequate to promote, or even permit, unrestricted differentiation.

Future investigation should determine how the tumor stem cells become oncogenic and how they are related to the mechanism of tumorigenesis in pituitary adenoma. Studies should also focus on how the tumor stem cells in adenomas can be a target of therapy. We believe that tumor stem cells offer a new target for medical therapies against refractory human pituitary adenomas.

In conclusion, this study demonstrated that human pituitary adenomas contain a small population of undifferentiated cells with stem-like properties. Because these cells demonstrate the ability to self-renew and differentiate into other cell types in a certain condition, we identified these cells as tumor stem cells. Furthermore, we found that $CD133^+$ is a potential marker for identifying and isolating tumor stem-like cells in human pituitary adenomas.

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Authors have no conflict of interest.

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