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# Isolation of adult pituitary stem/progenitor cell clusters located in the parenchyma of the rat anterior lobe

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ABSTRACT

Recent studies have demonstrated that *Sox2*-expressing stem/progenitor cells play roles in the pituitary cell turnover. Two types of niches have been proposed for stem/progenitor cells, the marginal cell layer (MCL) and the dense cell clusters in the parenchyma. Among them, the appearance of the parenchymal-niche only after birth indicates that this niche is involved in the cell turnover required for the postnatal pituitary. However, little is known about the roles of the parenchymal-niche and its regulation. The present study aimed to isolate pituitary stem/progenitor cells from the parenchymal-niche in the adult rat pituitary. Cell dispersion by stepwise treatment with proteases allowed the isolation of dense cell clusters. Immunocytochemistry demonstrated that clusters are universally composed of SOX2-positive cells, and most of them are positive for PROP1. Taken together with the anatomical analysis, we concluded that the isolated clusters are the parenchymal stem/progenitor cell (PS)-clusters, not the MCL-one. PS-clusters cultivated by serum-free overlay 3-dimensional culture maintained their stemness, and treatment with bFGF and EGF induced cyst-formation. Moreover, PS-clusters demonstrated some differentiation capacity with GSK3β-inhibitor treatment. Collectively, the present study demonstrates a simple method for isolating stem/progenitor cells from the parenchymal-niche, and provides tools to analyze the factors for regulating the pituitary niches.

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## 1. Introduction

The pituitary gland consists of two anatomically different entities. The adenohypophysis (anterior pituitary) is composed of the anterior and intermediate lobes, and the neurohypophysis of the posterior lobe. In particular, the anterior lobe plays key roles in maintaining biological homeostasis through synthesis and secretion of plural-hormones such as growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and adrenocorticotrophic hormone (ACTH). Although the turnover rate of pituitary hormone-producing cells can be as low as 1.58% per day (Levy, 2002; Levy, 2008; Nolan et al., 1998), the turnover of hormone-producing cells from stem/progenitor cells is important for sustaining the function of this endocrine tissue. Data obtained from an *in vitro* pituisphere forming assay and *in vivo* cell tracing using $Sox2^{CreERT2/+}/R26^{YFP/+}$ mice have demonstrated that stem/progenitor cells exist as non-hormone producing cells in the adult anterior pituitary, and that SOX2-positive cells are a primitive resource for hormone-producing cells (Andoniadou et al., 2013; Chen et al., 2009; Fauquier et al., 2008; Fu and Vankelecom, 2012; Fu et al., 2012; Rizzoti et al., 2013). Furthermore, we reported that SOX2-positive stem/progenitor cells constitute plural-populations defined by the existence of S100β, a pituitary-specific transcription factor PROP1 (Prophet of PIT1), and paired-related homeobox transcription factors PRRX1 and PRRX2 in the rat adult anterior pituitary (Higuchi et al., 2014; Yoshida et al., 2009; Yoshida et al., 2011). Notably, S100β could be an important factor for verifying the difference between pituitary embryonic and adult

*Abbreviations:* bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; CAR, coxsackievirus and adenovirus receptor; DAPI, 4,6-diamidino-2-phenylindole; ECM, extracellular matrix; EGF, epidermal growth factor; Ephrin, Eph family receptor interacting proteins; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GSK3β, glycogen synthase kinase 3β; KSR, knockout serum replacement; MCL, marginal cell layer; PI, propidium iodide; PIT1, pituitary-specific positive transcription factor 1; PS-cluster, parenchymal stem/progenitor cell-cluster; PROP1, prophet of PIT1; TBX19, T-box transcription factor 19; SF1, steroidogenic factor 1; SOX2, sex-determining region Y-box 2.

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stem/progenitor cells, because S100β-positive cells appear only after birth in the anterior pituitary (Soji et al., 1994; Yoshida et al., 2011).

A niche is defined as a microenvironment that maintains stem cells through growth factors, cell surface proteins and extracellular matrices (ECMs), and is identified in some organs such as the intestine, brain, skeletal muscle, skin and testis (Chen and Chuong, 2012; Gattazzo et al., 2014; Tanimura et al., 2011). In the adult anterior pituitary, the marginal cell layer (MCL) facing the residual lumen and SOX2-positive cell clusters scattered in the parenchyma of the anterior lobe are proposed as primary and secondary stem/progenitor cell niches, respectively, and the secondary niches originate from the primary niche (Gremeaux et al., 2012; Vankelecom and Chen, 2014). Interestingly, although the MCL-niche exists from early pituitary development to adult pituitary, the parenchymal-niche only appears after birth and increases in number during the early postnatal pituitary growth wave (Chen et al., 2013). In addition, our previous studies demonstrated that stem/progenitor cells construct both niches by a homophilic tight junction factor, CAR (coxsackievirus and adenovirus receptor encoded by the *Cxadr* gene) (Chen et al., 2013) and a juxtacrine factor, ephrin-B2 (Yoshida et al., 2015). We also showed that PROP1/SOX2-positive cells are significantly decreased in the MCL during the early postnatal pituitary growth wave, but are still maintained in the parenchymal-niche (Higuchi et al., 2014; Yoshida et al., 2011). Considering that S100β-positive stem/progenitor cells appear only after birth and the quantitative and qualitative transition of stem/progenitor cells occurs during early postnatal periods, the parenchymal-niche might play a role in the cell turnover required for the postnatal anterior pituitary.

A sphere-forming assay is a valuable method for analyzing the characteristics of stem/progenitor cells after forming a sphere originating from a single stem/progenitor cell in various tissues as well as in the pituitary (Chen et al., 2005; Fauquier et al., 2008; Gritti et al., 1996; Pastrana et al., 2011; Reynolds and Weiss, 1992). In addition, analysis of the side population, which is a fraction rich in stem/progenitor cells, have demonstrated several candidate regulators of stem/progenitor cells such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Chen et al., 2006; Chen et al., 2005; Fauquier et al., 2008). However, in the pituisphere and side population, it is difficult to distinguish whether SOX2-positive stem/progenitor cells are derived from the MCL-niche, the parenchymal-niche, or a single-SOX2 positive cell detached from the niches in the parenchyma. Therefore, isolation and characterization of the pituitary stem/progenitor cells of known origin provide useful information to clarify the mechanisms for regulating stem/progenitor cells in their niche.

In the current study, we aimed to isolate pituitary stem/progenitor cells from the parenchymal-niches by simple protease treatments. Stepwise protease dispersions of the anterior lobe of the adult rat pituitary allowed successful isolation and characterization of SOX2-positive stem/progenitor cell clusters. As immunocytochemistry clearly demonstrated that most clusters contain PROP1-positive cells, which primarily exist in the parenchymal-niche of the adult anterior lobe, we termed isolated-clusters “parenchymal stem/progenitor cell (PS)-clusters.” Based on the contents of S100β-positive cells, PS-clusters were classified into the three subtypes. Cultivation by the serum-free overlay 3-dimensional (3D) culture method prevented the progression of differentiation, and treatment with bFGF and EGF induced cyst-formation. Moreover, PS-clusters demonstrated some differentiation capacity with glycogen synthase kinase 3β (GSK3β)-inhibitor treatment. Taken together, we established a simple method for isolating the stem/progenitor cells from the pituitary parenchymal-niche.

## 2. Materials and methods

### 2.1. Animals

Wistar-crlj S100β/green fluorescent protein-transgenic rats (S100β/GFP-TG rats) generated by fusing the *S100β*-promoter to the reporter gene *GFP* (Itakura et al., 2007), and intact male Wistar-Imamichi rats were housed individually in a temperature-controlled room under a 12 h light/darkness cycle conditions. All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee, Meiji University, and were conducted in accordance with the NIH Guidelines for Animal Care and Use of Laboratory Animals.

### 2.2. Pituitary cell dispersion and isolation of dense cell clusters

Excised anterior lobes of the pituitary from 8- to 12-week-old male S100β/GFP-TG rats were chopped into six pieces and incubated in Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, Waltham, MA, USA) containing 0.2% collagenase (Sigma, St. Louis, MO USA) for 15 min at 37 °C, followed by incubation with DNase I (Promega, Madison, WI, USA) at 2 units/ml for 5 min at 37 °C. After removal of the collagenase solution by centrifugation at 100 $\times g$ for 5 min, collected cells were incubated in 10 mM HEPES-100 mM NaCl (pH 7.5; HEPES buffer) containing 0.25% trypsin (Sigma)-5 mM EDTA (Dojindo Laboratories, Kumamoto, Japan) for 10 min at 37 °C. After removal of the trypsin solution by centrifugation, collected cells were dispersed in HEPES buffer by vigorous pipetting and incubated in 0.75% $NH_4Cl$ in HEPES buffer for 3 min at room temperature to remove blood corpuscles. The cells were then suspended in a mixed medium (DMEM/F-12) composed of DMEM and Ham F-12 (both from Life Technologies, Grand Island, NY, USA) without serum. Cell suspension was plated on a non-adhesive 35-mm dish (AGC TECHNO GLASS, Shizuoka, Japan), and dense cell clusters were immediately collected manually using either pipettes under a microscope (Leica DM IRB, Leica, Wetzlar, Germany) or borosilicate glass capillaries approximately 100 μm in internal diameter under a microscope equipped with a controlled micromanipulator (Leica AM 6000, Leica). A cell viability test of dense cell clusters was performed using ReadyProbes Cell Viability Imaging Kit (Thermo Fisher Scientific). In an attempt to disperse collected dense cell clusters, additional treatment with 2.0% trypsin-5 mM EDTA in HEPES buffer for 30 min at 37 °C, and subsequent pipetting, was performed.

### 2.3. Cell culture

Collected dense cell clusters were cultured by the overlay 3D culture method (Debnath et al., 2003) using growth factor reduced Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA, USA). Briefly, 16-well chamber slides (0.4 $cm^2$/well) (Thermo Fisher Scientific) were coated with undiluted Matrigel (25 μl/$cm^2$), followed by seeding 1–5 dense cell clusters suspended in 1:50 diluted Matrigel in DMEM/F-12 without serum. To test the effects of growth factors, dense cell clusters were cultured by the overlay 3D culture method and 2D culture (described below) including 20 ng/ml each of recombinant mouse bFGF (R&D, Minneapolis, MN, USA) and recombinant human EGF (R&D) in DMEM/F-12 without serum. To assess the proliferative activity of dense cell clusters, bromodeoxyuridine (BrdU) (10 μM; Roche Diagnostics GmbH, Mannheim, Germany) was added to the culture medium at the time of cell seeding. Cultivation of dense cell clusters was carried out in a humidified atmosphere of 5% $CO_2$ and 95% air for 7 days, and time-dependent changes of each single cluster were photographed at 0, 1, 3, 5 and 7 days after seeding. Immunofluorescence was observed under the DMI6000 B inverted fluorescence microscope (Leica).

### 2.4. Growth of pituisphere

Growth of pituispheres was carried out according to previously reported conditions (Chen et al., 2005; Fauquier et al., 2008). Briefly, dispersed cells prepared from the anterior lobe as described above were cultured on 35-mm non-treated dishes (AGC TECHNO GLASS) at a density of 100,000 cells/ml in a DMEM/F-12 containing B27 supplement (1:50; Thermo Fisher Scientific), N2 supplement (1:100; Wako,

Osaka, Japan), BSA (0.5%; Sigma), bFGF (20 ng/ml) and EGF (20 ng/ml) for 5 days. On day 5, pituispheres were manually collected using pipettes under the Leica DM IRB microscope (Leica).

### 2.5. Differentiation assay

Differentiation assays for dense cell clusters and pituispheres were carried out using previously reported conditions for pituisphere (Chen et al., 2009; Fauquier et al., 2008). Briefly, dense cell clusters and pituispheres were manually collected and transferred to 16-well chamber slides coated with growth factor-reduced Matrigel diluted 1:10 in DMEM/F-12 without serum, and were cultured for 7 days. For another differentiation induction condition for dense cell clusters, the clusters were cultured by the overlay 3D culture method with 20 ng/ml each of bFGF and EGF, and 20% KnockOut Serum Replacement (KSR) (Thermo Fisher Scientific) for 4 days, followed by replacement with medium including 6-bromoindirubin-3′-oxime (BIO) (GSK3β-inhibitor, 250 nM; Wako) and cultivation for another 7 days.

### 2.6. Immunocytochemistry

For immunocytochemistry, manually collected dense cell clusters without cultivation were mounted on a MAS-coat glass slide (Matsunami Glass, Osaka, Japan) using Smear Gel (Genostaff, Tokyo, Japan) as necessary. Dense cell clusters and pituispheres cultured by the overlay 3D culture method or on Matrigel-coated glass slide were washed with HEPES buffer. Cells were fixed with 4% or 1% paraformaldehyde in 20 mM HEPES, pH 7.5, for 30 min at room temperature or 10 min at 4 °C, or with 95% ethyl alcohol for 30 min at −20 °C, followed by washing with HEPES buffer. Cells were antigen-retrieved using an ImmunoSaver (0.05% citraconic anhydride solution, pH 7.4; Nisshin EM, Tokyo, Japan) as necessary (Supplementary Table 1). For BrdU-staining, cells were treated with 1 N HCl in HEPES buffer for 10 min at room temperature. Cells were incubated with blocking buffer containing 10% (v/v) FBS and 0.4% (v/v) Triton ×100 in HEPES buffer for 60 min at room temperature. Reaction with primary antibodies was performed at an appropriate dilution (Supplementary Table 1) with blocking buffer overnight at room temperature or 4 °C. To detect hormone-positive cells, a cocktail of anti-hormone antibodies at an appropriate dilution (Supplementary Table 1) was used. After immunoreaction and washing with HEPES buffer, cells were incubated with secondary antibodies using Cy3-, Cy5-, or FITC-conjugated AffiniPure donkey anti-goat, rabbit, mouse and guinea pig IgG, as well as chicken IgY (1:500; Jackson ImmunoResearch, West Grove, PA, USA). Cells were washed and incubated with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA) with DAPI (4, 6′-diamidino-2-phenylindole, dihydrochloride). Immunofluorescence was observed under the DMI6000 B inverted microscope (Leica) and the confocal fluorescence microscope, FLUOVIEW FV1000 (Olympus, Tokyo, Japan).

### 2.7. Immunohistochemistry

The pituitaries of male postnatal day 60 (P60) Wistar-Imamichi rats were fixed in 4% paraformaldehyde in 20 mM HEPES, overnight at 4 °C, followed by immersion in 30% trehalose in 20 mM HEPES to cryoprotect the tissues. They were embedded in Tissue-Tek O.C.T compound (Sakura Finetek Japan, Tokyo, Japan) and frozen immediately. Frozen sections (7 μm thick) from the coronal plane were prepared. The sections were antigen-retrieved by an ImmunoSaver for 1 h at 80 °C. After washing with HEPES buffer, these sections were reacted with primary antibodies at appropriate dilutions (Supplementary Table 1) with blocking buffer overnight at 4 °C. After the immunoreaction, sections were washed with HEPES buffer, and then incubated with secondary antibodies using Cy3-, Cy5-, or FITC-conjugated AffiniPure donkey *anti*-mouse, rabbit, goat and guinea pig IgG (1:500 dilution). The sections were washed with HEPES buffer and then enclosed in VECTASHIELD Mounting Medium with DAPI. Immunofluorescence was observed under the BZ-8000 fluorescence microscope (KEYENCE, Osaka, Japan).

### 2.8. Statistical analysis

Cyst-formation data and the proportion of dense cell clusters were validated using the *F*-test and data showing normal distribution were analyzed using the Student's *t*-test.

## 3. Results

### 3.1. Immunohistochemical characterization of the proposed pituitary stem/progenitor cell niches in the anterior lobe of the adult rat pituitary

In the adult anterior pituitary, two types of stem/progenitor cell niches, the MCL-niche and parenchymal-niche, have been proposed (Chen et al., 2013; Gremeaux et al., 2012; Vankelecom and Chen, 2014). Although the MCL-niche is maintained from early pituitary development, the parenchymal-niche appears only after birth and increases in number over time (Chen et al., 2013). To analyze their morphology and characteristics, we performed triple-immunostaining for SOX2, E-cadherin and a pituitary specific progenitor cell marker, PROP1 (Yoshida et al., 2009; Yoshida et al., 2015; Yoshida et al., 2011) in the rat pituitary of P60 (Fig. 1). Immunostaining for SOX2 and E-cadherin, a cell-cell adhesion molecule used as the pituitary stem/progenitor cell marker (Fauquier et al., 2008), demonstrated that both proposed niches were composed of cells double-positive for SOX2 and E-cadherin (Fig. 1). Especially, in the parenchymal-niche, SOX2-positive cells were closely connected by E-cadherin and formed dense cell clusters (Fig. 1c, c′). Immunostaining for PROP1 demonstrated that PROP1-positive cells existed at low frequency in the MCL-niche of the adult pituitary (Fig. 1b, b′, arrowheads). On the other hand, most of the parenchymal-niche contained PROP1-positive cells (Fig. 1c, c′) as shown in our previous studies (Yoshida et al., 2015; Yoshida et al., 2011).

Next, we performed immunohistochemistry for pituitary lineage specific-commitment cell markers, PIT1 (GH-, PRL-, TSH-lineage), TBX19 (also known as TPIT; ACTH-lineage) and SF1 (LH- and FSH-lineage) in both niches of the adult pituitary (Supplementary Fig. 1). Triple-immunostaining demonstrated that each PIT1-, TBX19- and SF1-positive cell located outside both niches but not in either the MCL-niche (Supplementary Fig. 1a–c) or the parenchymal-niche (Supplementary Fig. 1d–f).

### 3.2. Characterization of dense cell clusters collected from the dispersed anterior lobe of the adult rat pituitary

Since some niches are composed of unique cell surface proteins and ECMs such as heparin sulfate- and chondroitin sulfate-proteoglycans, collagen, tenascin-C and other glycosaminoglycans (Gattazzo et al., 2014; Hayashi et al., 2009; Itano et al., 2008; Tanimura et al., 2011), we hypothesized that stem/progenitor cell niches have a structure resistant to treatment with both protease and mechanical dispersion. Hence, we examined stepwise treatment with collagenase and trypsin, followed by vigorous pipetting. We identified a number of dense cell clusters in the dispersed cell suspension of the anterior lobe (Fig. 2a–d′). They were easy to distinguish from cell clumps formed by loosely aggregated cells (Fig. 2a, arrowheads).

These dense cell clusters were collected manually using pipettes or borosilicate glass capillaries. Collected dense cell clusters could not be dispersed by additional treatment with a higher concentration of 2.0% trypsin-5 mM EDTA for 30 min at 37 °C and subsequent pipetting (data not shown). Collected dense cell clusters were mainly ellipse-shaped and composed of small cells (approximately 7.5 μm in diameter). The diameter of the major axis of clusters was 28.6 ± 5.1 μm (means ± SD from forty-nine dense cell clusters) ranging from 18.6–42.5 μm

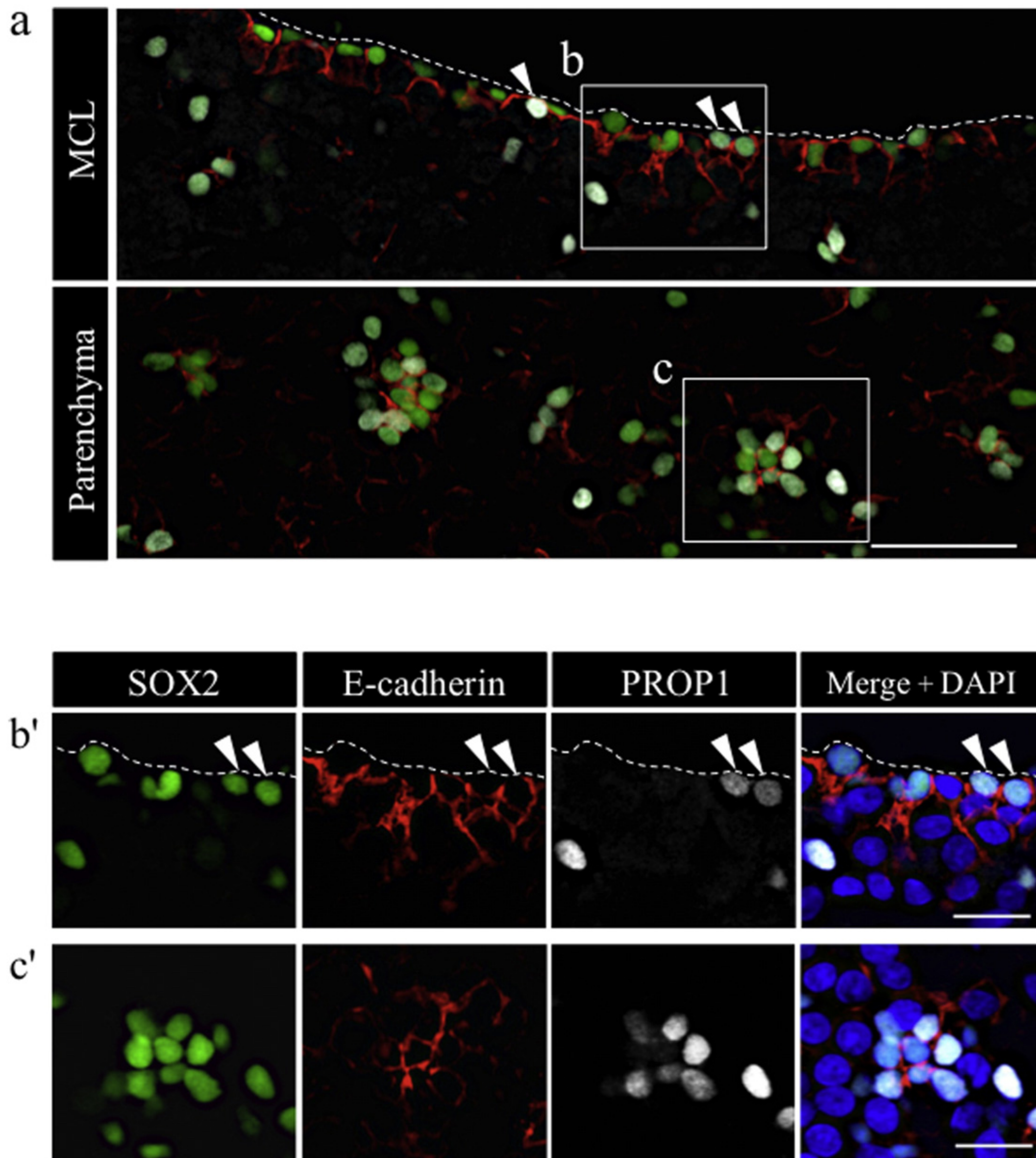
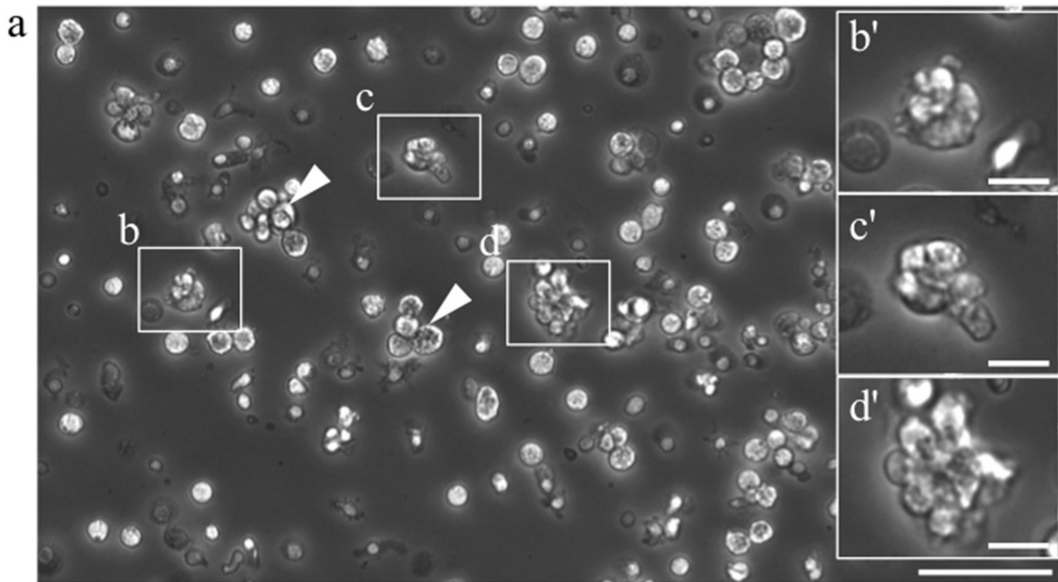



**Fig. 1.** Immunohistochemistry of the MCL- and parenchymal-niches in the adult rat anterior lobe. Triple-immunostaining for SOX2, E-cadherin and PROP1 using the pituitary of P60 rat was performed. a Merged images of SOX2 visualized with FITC (*green*), E-cadherin with Cy3 (*red*) and PROP1 with Cy5 (*white*) for the MCL (upper panel) and parenchyma (lower panel) of the anterior lobe are shown. SOX2, E-cadherin, PROP1 and a merged image with nuclear staining by DAPI (*blue*) in boxed areas in the MCL-niche (b) and parenchymal-niche (c) are enlarged in (b′) and (c′), respectively. *Dotted lines* indicate the border of the marginal cell layer. *Arrowheads* indicate PROP1-positive cells in the MCL. *Bars* 50 μm (a), 20 μm (b′ and c′).

(Fig. 2a–d′), and a single dense cell cluster consisted of about 12 ± 6 cells (means ± SD from 24 dense cell clusters), as estimated by DAPI staining. Since about 76 dense cell clusters were observed in approximately $1.1 \times 10^4$ dispersed cells (Fig. 2e), it was calculated that about $1.4–2.1 \times 10^4$ of dense cell clusters were present per single adult anterior lobe (composed of about $2–3 \times 10^6$ cells).

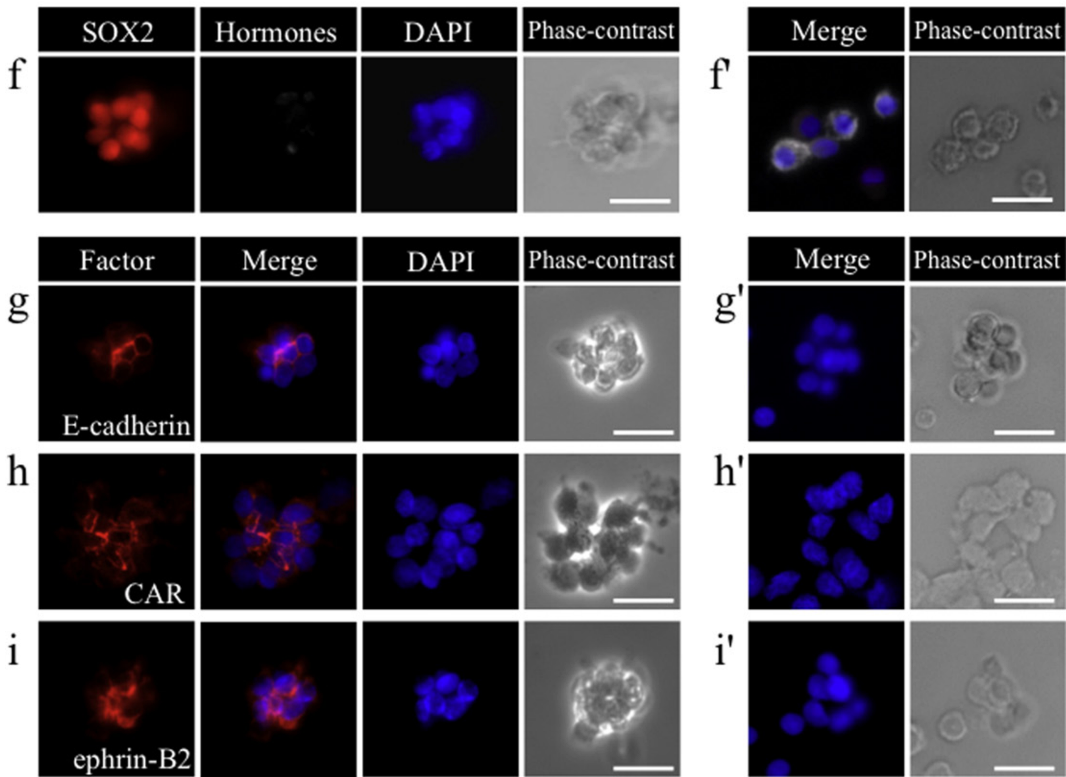
### 3.3. Immunocytochemistry of dense cell clusters

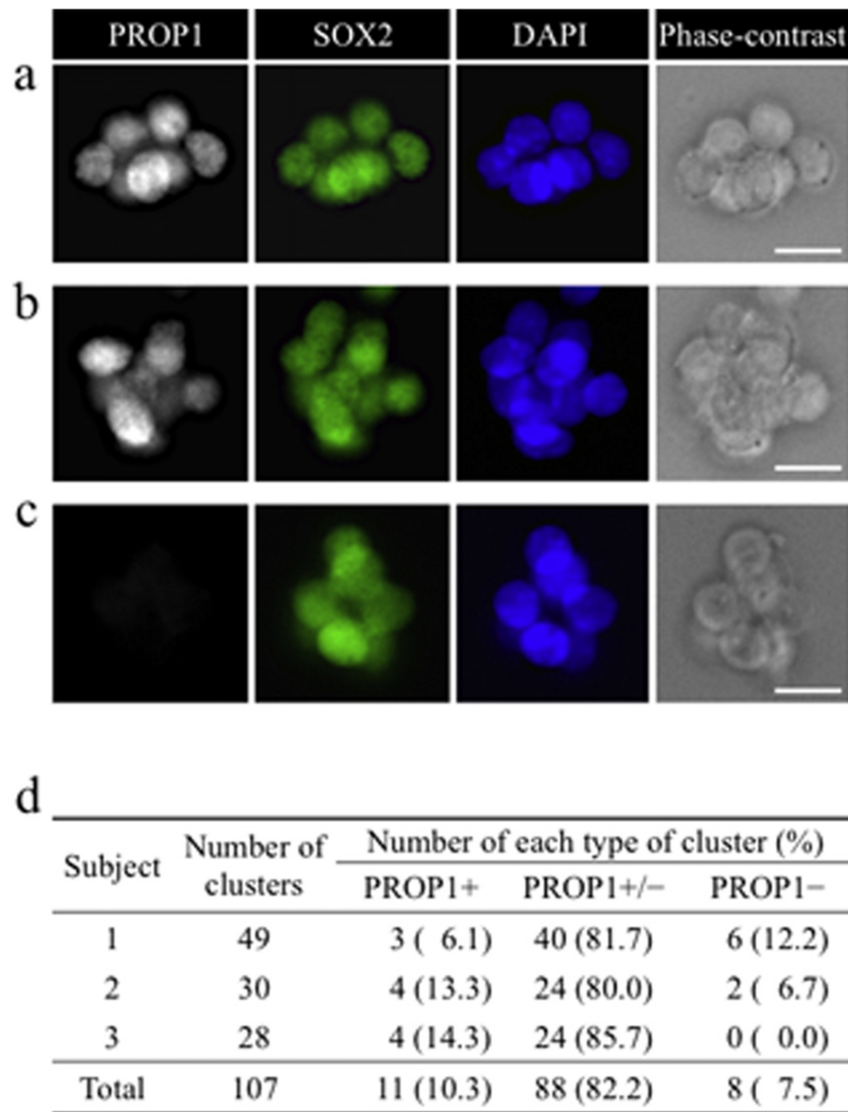
Double-immunostaining for SOX2 and pituitary hormones (using a cocktail of antibodies against GH, TSHβ, PRL, LHβ, FSHβ, αGSU and ACTH) were performed to characterize dense cell clusters. Typical staining images are shown in Fig. 2f–i′. Double-immunostaining demonstrated that dense cell clusters were universally composed of SOX2-positive (Fig. 2f) but hormone-negative cells, whereas cells in clumps were mainly SOX2-negative but hormone-positive (Fig. 2f′). Since all of the cells in the 66 dense cell clusters examined were positive for SOX2, randomly collected dense cell clusters were further characterized using several cell markers (Fig. 2g–i′). E-cadherin was first examined, and intense E-cadherin signals were observed at the intercellular surface whereas faint signals were observed at the peripheral surface (Fig. 2g). Intense signals of CAR, a cell-surface receptor protein that localizes in both of the pituitary stem/progenitor cell niches (Chen et al., 2013), were observed at the intercellular surface in a manner similar to E-cadherin (Fig. 2h). Immunocytochemistry of ephrin-B2, which also localizes in both pituitary stem cell niches (Yoshida et al., 2015), showed a pattern similar to that of CAR (Fig. 2i). However, cell clumps were negative for E-cadherin, CAR and ephrin-B2 (Fig. 2g′–i′). Moreover, immunocytochemistry demonstrated that cells positive for commitment cell markers, PIT1, TBX19 or SF1 did not locate in dense cell clusters (Supplementary Fig. 2a–c), but located in cell clumps or the single cells among dispersed cells (Supplementary Fig. 2a′–c′). This




e

| Subject | Cell number counted | Number of clusters |
|---|---|---|
| 1 | 11926 | 69 |
| 2 | 10833 | 82 |
| AVE. | 11380 | 76 |






| Subject | Number of clusters | Number of each type of cluster (%) | | |
|---|---|---|---|---|
| | | PROP1+ | PROP1+/− | PROP1− |
| 1 | 49 | 3 ( 6.1) | 40 (81.7) | 6 (12.2) |
| 2 | 30 | 4 (13.3) | 24 (80.0) | 2 ( 6.7) |
| 3 | 28 | 4 (14.3) | 24 (85.7) | 0 ( 0.0) |
| Total | 107 | 11 (10.3) | 88 (82.2) | 8 ( 7.5) |

**Fig. 3.** Immunocytochemistry for the pituitary progenitor cell marker PROP1 in dense cell clusters. a–c PROP1 visualized with Cy3 (white), SOX2 with FITC (green), nuclear staining by DAPI (*blue*) and phase-contrast are shown. According to the content of PROP1-positive cells, dense cell clusters were classified into three types, PROP1+ (all PROP1-positive cells; a), PROP1+/− (mixture of PROP1-positive and -negative cells; b) and PROP1− (all PROP1-negative cells; c). *Bars* 10 μm. d Proportion of dense cell clusters based on the composition of PROP1-positive cells. In the PROP1+/− -cluster, the proportion of PROP1-positive cells was 65.6 ± 20% (means ± SD for 24 PROP1+/− -clusters). Subject indicates independent animals.

correlated with our immunohistochemical results (Supplementary Fig. 1). These data indicate that these dense cell clusters are originated from the pituitary stem/progenitor cell niches.

Finally, immuno-positive signals of PROP1, which primarily exists in the parenchymal-niche of the adult rat anterior lobe (Yoshida et al., 2015; Yoshida et al., 2011), were found in about 92% of the dense cell clusters examined (Fig. 3). In addition, we confirmed that these dense cell clusters were also able to be isolated from the most ventral-lateral region of the anterior lobe, which does not include the MCL (Supplementary Fig. 3b, arrowheads), in the same proportion as the anterior lobe including the MCL (Supplementary Fig. 3c). Considering that the morphology of isolated dense cell clusters is similar to that of the parenchymal-niche, we concluded that these dense cell clusters were originated from the parenchymal-niches. We therefore termed them the "parenchymal stem/progenitor cell-cluster (PS-cluster)."

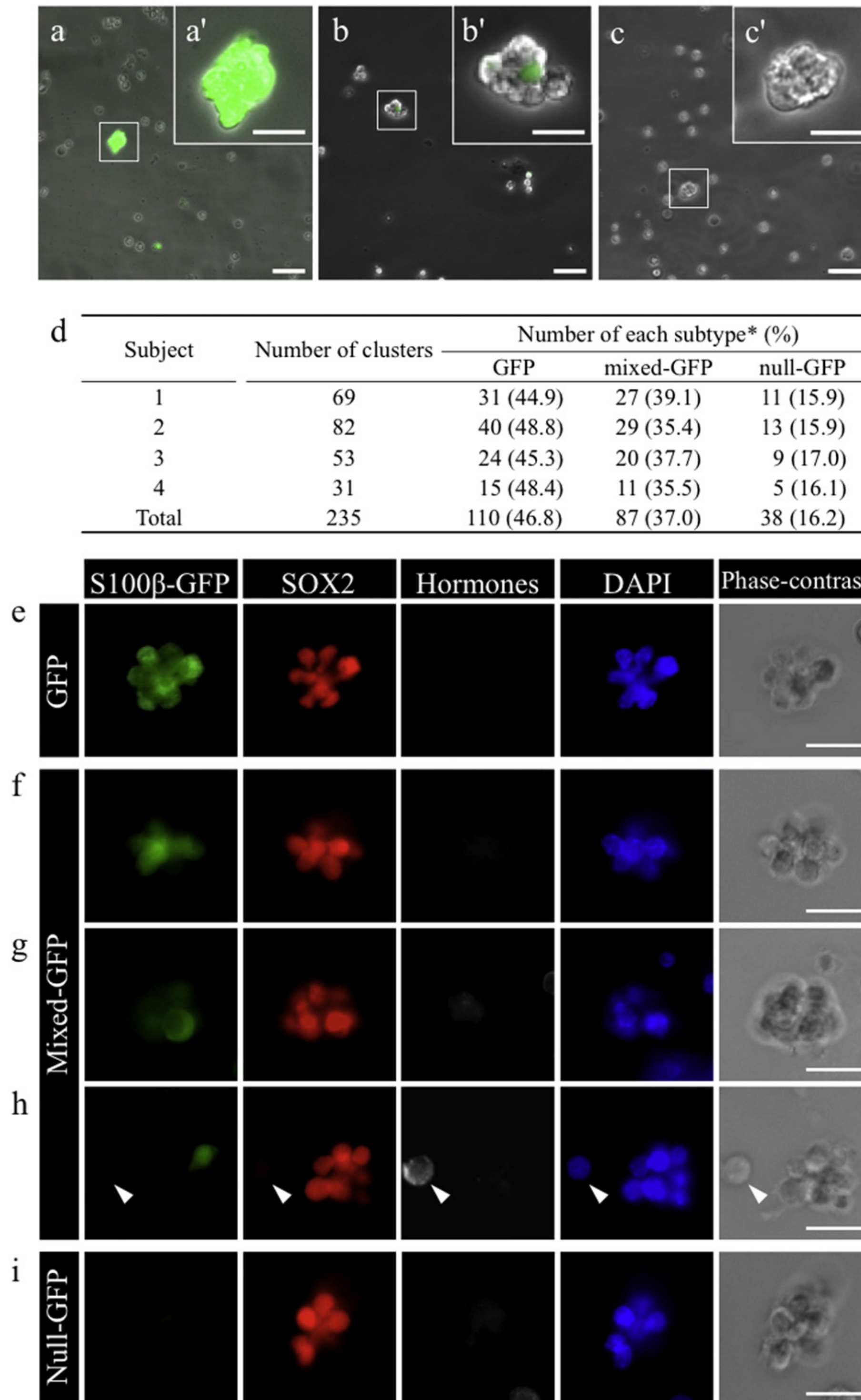
Next, we classified the PS-clusters into three subtypes based on the contents of S100β-GFP-positive cells: GFP-cluster (all composed of GFP-positive cells) (Fig. 4a, a′), mixed-GFP-cluster (composed of GFP-positive and -negative cells) (Fig. 4b, b′) and null-GFP-cluster (all

**Fig. 2.** Dense cell clusters found among dispersed cells prepared from the adult rat anterior lobe. a–d′ Phase-contrast image of dispersed cells prepared from the anterior lobes of the adult rat pituitary is shown. Dense cell clusters in boxed areas (b–d) are enlarged (b′–d′), respectively. *Arrowheads* in a indicate cell clumps. e Number of dense cell clusters in the dispersed anterior lobe of the rat pituitary gland. "Cell number counted" indicates the sum of dispersed cells and the cells in dense cell clusters. The number of cells composing a single dense cell cluster is estimated as 12 (means from 24 dense cell clusters as estimated by DAPI staining). Subject indicates independent animals. f, f′ Immunocytochemistry for SOX2 and pituitary hormones using a cocktail of anti-hormone antibodies in dense cell clusters (f) and cell clumps (f′) was performed. SOX2 visualized with Cy3 (*red*), pituitary hormones with Cy5 (*white*), nuclear staining by DAPI (*blue*) and phase-contrast are shown in f. Merged image of SOX2, pituitary hormones and nuclear staining, and phase-contrast are shown in (f′). g–i′ Immunocytochemistry for pituitary stem/progenitor cell niche markers, E-cadherin (g, g′), CAR (h, h′) and ephrin-B2 (i, i′) in dense cell clusters (g–i) and cell clumps (g′–i′) was performed. Each factor visualized with Cy3 (*red*), nuclear staining by DAPI (*blue*) and the merged images is shown. *Bars* 50 μm (a), 10 μm (b′–d′), 20 μm (f–i′).

composed of GFP-negative cells) (Fig. 4c, c′). The proportion of those subtypes, i.e., GFP-, mixed-GFP- and null-GFP-clusters, were about 47, 37 and 16%, respectively (Fig. 4d). Immunocytochemistry demonstrated that all types of PS-clusters were commonly composed of SOX2-positive and hormone-negative cells regardless of S100β-GFP signals, as shown in Fig. 4e–i.

### *3.4. Cultivation of PS-clusters by the overlay 3D culture method in the presence and absence of growth factors*

To establish a culture method for maintaining the 3D structure and undifferentiated state of the PS-clusters, we cultivated PS-clusters of each subtype by an overlay 3D culture method using a growth factor




| Subject | Number of clusters | Number of each subtype* (%) | | |
|---|---|---|---|---|
| | | GFP | mixed-GFP | null-GFP |
| 1 | 69 | 31 (44.9) | 27 (39.1) | 11 (15.9) |
| 2 | 82 | 40 (48.8) | 29 (35.4) | 13 (15.9) |
| 3 | 53 | 24 (45.3) | 20 (37.7) | 9 (17.0) |
| 4 | 31 | 15 (48.4) | 11 (35.5) | 5 (16.1) |
| Total | 235 | 110 (46.8) | 87 (37.0) | 38 (16.2) |

**Fig. 4.** Immunocytochemistry for S100β-GFP in PS-clusters. Merged images of GFP (*green*) and phase-contrast for PS-clusters composed of GFP-positive cells (GFP-cluster; a, a′), mixture of GFP-positive and -negative cells (mixed-GFP-cluster; b, b′), and GFP-negative cells (null-GFP-cluster; c, c′) in dispersed cells from the anterior lobe are shown. d The proportion of three types of PS-clusters based on the composition of S100β-GFP-positive cells in PS-clusters. Subject indicates independent animals. e–i Triple-immunostaining for GFP, SOX2 and pituitary hormones in the GFP-cluster (e), mixed-GFP-cluster (f-h) and null-GFP-cluster (i) was performed. GFP visualized with FITC (*green*), SOX2 with Cy3 (*red*) and pituitary hormones with Cy5 (*white*), nuclear staining by DAPI (*blue*) and phase-contrast are shown. *Arrowheads* indicate a SOX2/GFP-double negative/hormone-positive cell present in extra-PS-cluster. *Bars* 50 μm (a–c), 20 μm (a′–c′, e–i).

reduced Matrigel in serum-free DMEM/F-12 for 7 days. Prior to cultivation, using propidium iodide (PI)-staining, we confirmed that all subtypes of PS-clusters were composed of living cells (Supplementary Fig. 4). During 7-day culture, most GFP- (Fig. 5a, b) and mixed-GFP-clusters (Fig. 5c) held tightly compacted morphologies with a smooth outer layer, whereas most null-GFP-clusters died (78%, 14 of 18 null-GFP-clusters) (Fig. 5d). In GFP-clusters, S100β-GFP signals were maintained (Fig. 5a) or slightly reduced (Fig. 5b) during cultivation. To confirm whether an undifferentiated state was maintained, we performed immunocytochemistry for the GFP- and mixed-GFP-clusters after 7-day cultivation. We observed that all of the cells composing PS-clusters were positive for SOX2 but negative for hormones (18 GFP- and 12 mixed-GFP-clusters examined) (Fig. 5a′–c′) even after 7-day cultivation. On the other hand, in rat pituispheres (Chen et al., 2005; Fauquier et al., 2008), hormone-positive cells appeared in this condition using the overlay 3D culture method (Supplementary Fig. 5).

Next, to examine the effect of growth factors, we cultured GFP- and mixed-GFP clusters in the presence of bFGF and EGF, which are shown as inducers for proliferation of pituitary side-population cells (Chen et al., 2006), cyst-like structure formation for cells of anterior lobe in aggregate cell culture (Krylyshkina et al., 2005) and pituisphere formation (Chen et al., 2005; Fauquier et al., 2008). During cultivation by the overlay 3D culture method with bFGF and EGF, about 89% (22 of 25 GFP-clusters) and 76% (15 of 22 mixed-GFP-clusters) of the GFP- and mixed-GFP-clusters formed a cyst-structure, respectively (Fig. 6a–c, Supplementary Fig. 6 and Supplementary Movie 1), whereas this structure was hardly observed in the absence of bFGF and EGF (0 of 15 GFP-clusters and 1 of 20 mixed-GFP-clusters) (Fig. 5a–c and Fig. 6c). Next, we confirmed the effect of bFGF and EGF on proliferation (Fig. 6d–f). Immunostaining for BrdU after 3D cultivation demonstrated that PS-clusters (15 GFP-clusters and 10 mixed-GFP-clusters examined) were negative for BrdU in the presence of bFGF and EGF (Fig. 6e, f) as well as in the absence of bFGF and EGF (18 PS-clusters examined) (Fig. 6d). In contrast to 3D cultivation, after 7-day 2D-cultivation on Matrigel-coated glass slides in the presence of bFGF and EGF, the cells derived from PS-clusters actively proliferated (Supplementary Fig. 7b, c). BrdU-positive signals were observed in most of the cells derived from both GFP- and mixed-GFP-clusters (71% and 77%, respectively), while BrdU-positive cells were hardly observed in the absence of bFGF and EGF (14% and 11% of the cells derived from GFP- and mixed-GFP-clusters, respectively) (Supplementary Fig. 7a, d). These differences between 3D- and 2D-cultivation might be caused by differences in cell-to-cell and cell-to-ECM interactions.

### *3.5. Analysis of the differentiation capacity of PS-clusters compared to the pituisphere*

Next, we examined the differentiation capacity of PS-clusters by a method established for the pituisphere using Matrigel-coated glass slides and serum-free medium (Chen et al., 2009; Fauquier et al., 2008). After 7-day cultivation in differentiation condition, immunocytochemistry

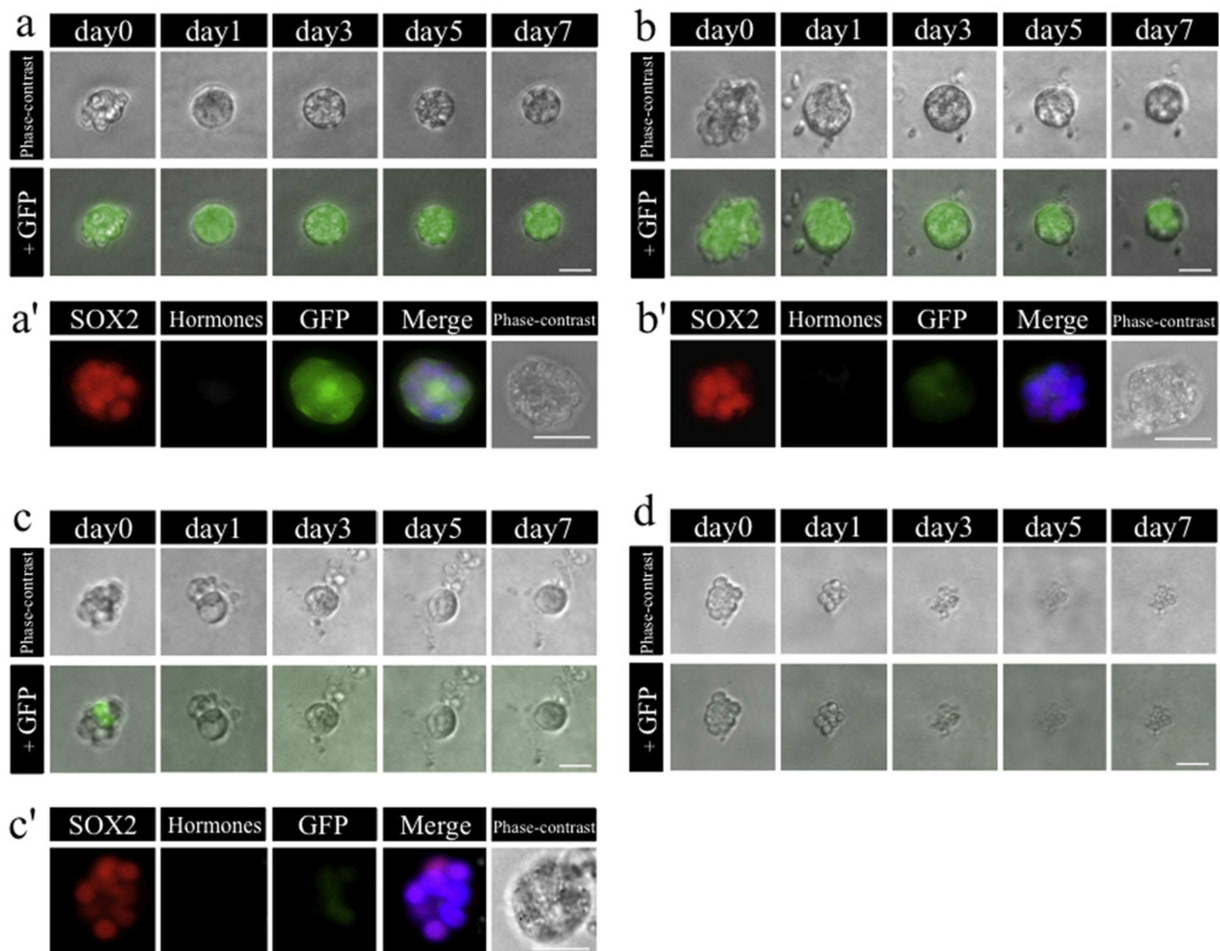



**Fig. 5.** 3D cultivation and immunocytochemistry of PS-clusters. a–d Time-lapse images of GFP- (a, b), mixed-GFP- (c) and null-GFP-clusters (d) during 3D cultivation by the overlay 3D culture method are shown. Phase-contrast (upper panels) and merged images with fluorescence (lower panels) of 3D cultured PS-clusters are shown. Images were obtained at 0, 1, 3, 5 and 7 days after seeding. a′–c′ Triple-immunostaining for SOX2, pituitary hormones and GFP after 7-day 3D culture in GFP- (a′, b′) and mixed-GFP-clusters (c′) was performed. Images of SOX2 visualized with Cy3 (*red*), pituitary hormones with Cy5 (*white*) and GFP with FITC (*green*), merged images with nuclear staining by DAPI (*blue*), and phase-contrast are shown. *Bars* 20 μm.

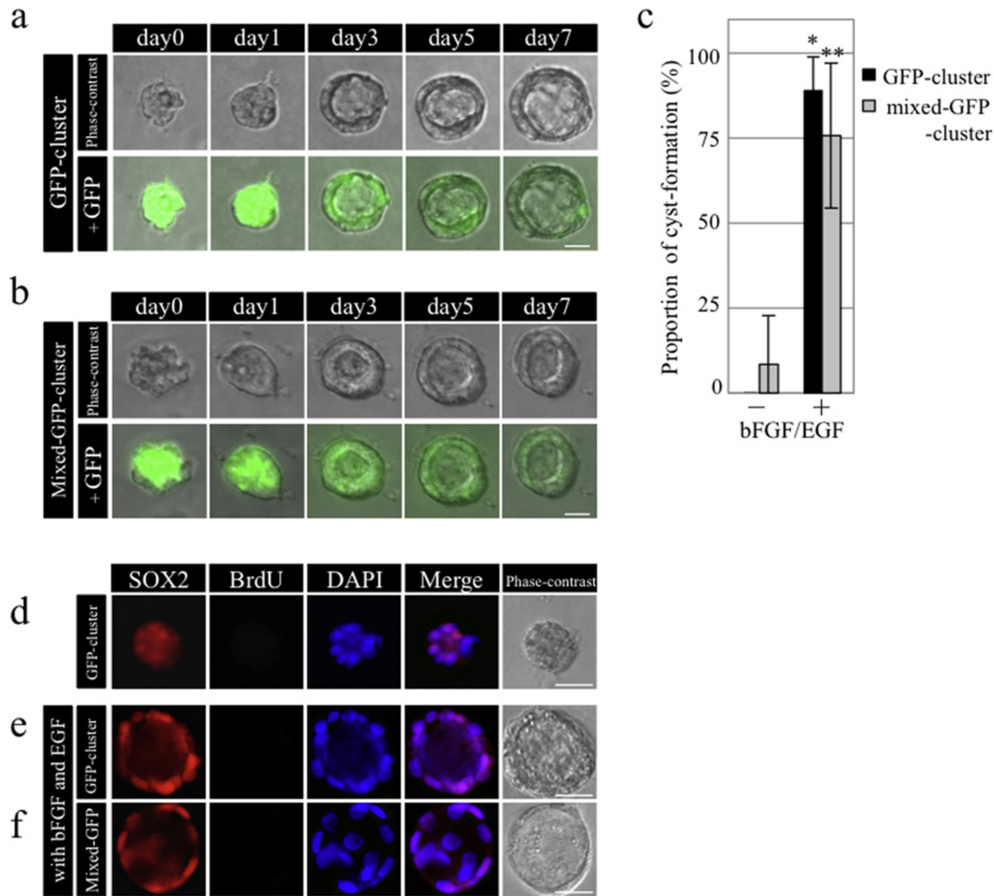



**Fig. 6.** 3D cultivation of PS-clusters in the presence of bFGF and EGF. a, b Cyst-forming activity of GFP- (a) and mixed-GFP-clusters (b) during 3D cultivation with bFGF and EGF by the overlay 3D culture method are shown. Phase-contrast (upper panels) and merged images with fluorescence (lower panels) of 3D cultured PS-clusters are shown. Images were obtained at 0, 1, 3, 5 and 7 days after seeding. c Proportion of cyst-formation in PS-clusters after 7-day cultivation with or without bFGF and EGF. Each black and gray bar indicates GFP- and mixed-GFP-clusters, respectively. The data are presented as the mean ± SD in three independent experiments. *$P < 0.01$, **$P < 0.05$. d–f Proliferation activity of PS-clusters was analyzed by 3D cultivation in the presence of bFGF and EGF. Double-immunostaining for BrdU and SOX2 in GFP- (d, e) and mixed-GFP-clusters (f) after 7-day 3D cultivation in the absence (d) and presence of bFGF and EGF (e, f) was performed. SOX2 visualized with Cy3 (*red*), BrdU with Cy5 (*white*), nuclear staining by DAPI (*blue*), merged images with nuclear staining and phase-contrast are shown. *Bars* 20 μm.

demonstrated that most of rat pituispheres maintained their 3D structure, and cells double-positive for SOX2 and pituitary hormones appeared in the sphere (Fig. 7a). However, PS-clusters attached on Matrigel-coat, and immunocytochemistry after 7-day cultivation demonstrated that all of the cells composing PS-clusters were SOX2-positive but hormone-negative (34 PS-clusters examined) (Fig. 7b).

Finally, we developed one condition for differentiation of PS-clusters (Fig. 7c). Taken together with reports about treatment of bFGF and EGF (Chen et al., 2006; Chen et al., 2009; Fauquier et al., 2008; Krylyshkina et al., 2005) and data in this study (Fig. 6 and Supplementary Fig. 7), bFGF and EGF are assumed to activate and be essential for maintaining pituitary stem/progenitor cells. Therefore, after pre-treatment with bFGF, EGF and 20% KSR by overlay 3D culture for 4 days, these PS-clusters were cultured for another 7 days in the presence of bFGF, EGF, KSR and GSK3β inhibitor, BIO, which is reported as an inducer of differentiation of Rathke's pouch-like vesicles derived from mouse ES cells into PIT1-lineage cells (Suga et al., 2011). Immunocytochemistry using a cocktail of anti-hormone antibodies after cultivation demonstrated that SOX2/hormone-double positive cells appeared in both GFP- and mixed-GFP-clusters at a frequency of about 18.8% (13 of 69 PS-clusters) (Fig. 7c and Supplementary Table 2). The average proportion of hormone-positive cells found in the PS-cluster, which comprise 14 ± 4 cells (means ± SD), was 16 ± 6% (means ± SD). However, without BIO or pre-treatment with bFGF, EGF and KSR, hormone-positive cells were observed in PS-clusters at a lower frequency of approximately 1.2% (1 of 83 PS-clusters) and 4.3% (2 of 47 PS-clusters), respectively (data not shown). In contrast to 3D-cluture, we could not develop more effective differentiating conditions for hormone-producing cells in 2D culture (data not shown). In 3D culture, immunocytochemistry using each anti-hormone antibody demonstrated that at least GH-positive cells appeared in PS-clusters at a frequency of about 3.1% (4 of 127 PS-clusters) (Fig. 7d and Supplementary Table 2). Moreover, using a cocktail of anti-hormone antibodies except for GH, we demonstrated that other types of hormone-positive cells also appeared at a frequency of about 9.5% (2 of 21 PS-clusters) (Fig. 7e and Supplementary Table 2). In order to detect all types of hormone-positive cells using each antibody, we need to increase the number of PS-clusters in each analysis or develop more effective differentiating conditions. Therefore, the issue of whether PS-clusters can produce all types of hormone-producing cells or limited ones remains to be elucidated.

## 4. Discussion

Recent reports proposed that SOX2-positive stem/progenitor cells of the adult anterior lobe form two types of niche, the MCL and the scattered dense cell clusters in the parenchyma (Chen et al., 2013; Gremeaux et al., 2012; Yoshida et al., 2015). Especially, the appearance of the parenchymal-niche only after birth (Chen et al., 2013) indicates that the parenchymal-niche plays key roles in the cell turnover required

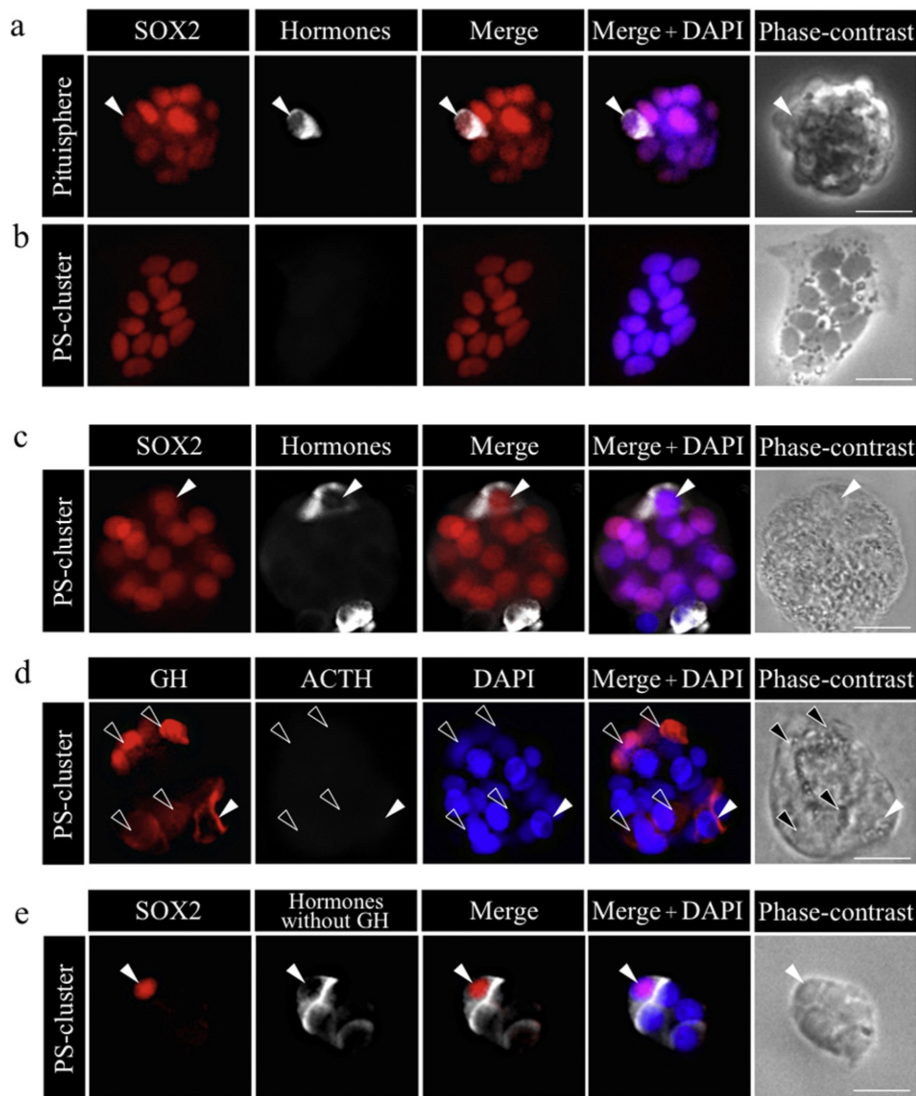



**Fig. 7.** Differentiation capacity of the PS-cluster and pituisphere. Immunocytochemistry for SOX2 and pituitary hormones of pituisphere (a) and PS-cluster (b) after differentiation induction on growth factor-reduced Matrigel-coated glass slides for 7 days was performed. SOX2 visualized with Cy3 (*red*), pituitary hormones with Cy5 (*white*), merged images without or with nuclear staining by DAPI (*blue*), and phase-contrast are shown. *Arrowheads* indicate cells double-positive for SOX2- and pituitary hormone. c–e Immunocytochemistry of PS-cluster after differentiation induction by the overlay-3D culture method in the presence of bFGF, EGF, KSR and GSK3β-inhibitor (BIO). After formation of the cyst-structure by the overlay 3D culture method for 4 days in medium containing bFGF, EGF and KSR, PS-clusters were cultured for another 7 days in the presence of BIO including bFGF, EGF and KSR. SOX2 visualized with Cy3 (*red*), pituitary hormones with Cy5 (*white*), merged images without and with nuclear staining by DAPI (*blue*), phase-contrast are shown in (c). GH visualized with Cy3 (*red*), ACTH with Cy5 (*white*), merged images without and with nuclear staining by DAPI (*blue*) and phase-contrast are shown in (d). SOX2 visualized with Cy3 (*red*), pituitary hormones except GH with Cy5 (*white*), merged images without and with nuclear staining by DAPI (*blue*), phase-contrast are shown in (e). *Arrowheads* in (a, c and e) indicate cells double-positive for SOX2 and pituitary hormones. The *closed*- and *open-arrowheads* in (d) indicate cells positive for GH that were in focus and located in a different z-axis position, respectively. *Bars* 20 μm.

for the postnatal anterior pituitary. However, this hypothesis has not been verified, because we lacked appropriate methods for isolating the pituitary stem/progenitor cells from the parenchymal-niche. In this study, we aimed to isolate stem/progenitor cells from the parenchymal-niche and succeeded in isolating the dense cell clusters originating from the parenchymal-niche, termed PS-clusters, by taking advantage of its tight structure resistant to protease treatment. The PS-clusters were composed of three subtypes based on S100β-GFP. Their stemness was confirmed by the presence of several stem/progenitor cell markers and their differentiation capacity by *in vitro* differentiation assay.

In the parenchymal-niche found in the adult anterior lobe, SOX2-positive cells were more densely connected to each other than in the MCL-niche, and formed dense cell clusters by E-cadherin (Fig. 1). We previously demonstrated that CAR and ephrin-B2 specifically localize at the apical cell membrane and intercellular surface of the dense cell clusters in the parenchyma, while in the MCL, they mainly localize at apical cell membranes facing the lumen (Chen et al., 2013; Yoshida et al., 2015). Further analyses demonstrated that, in the adult pituitary, PROP1/SOX2-double positive cells compose approximately 5.8% of SOX2-positive cells in the MCL, whereas about 90% of the parenchymal-niches of the anterior lobe contain PROP1-positive cells (Yoshida et al., 2015; Yoshida et al., 2011). Immunocytochemical images of E-cadherin, CAR and ephrine-B2 in the PS-clusters corresponded well to histological images observed in the parenchyma of the anterior lobe. Notably, we clearly showed that about 92% of PS-clusters contain PROP1-positive stem/progenitor cells. Taken together with a similarity of morphology between the isolated-dense cell clusters and parenchymal-niche, we concluded that dense cell clusters isolated in this study are originated from the parenchymal-niche, not the MCL-niche in the anterior lobe.

Although both the MCL- and parenchymal-niches are similarly composed of SOX2- and E-cadherin-positive cells, the present method using trypsin and collagenase isolated only the stem/progenitor cells from the parenchymal-niche, not the MCL-niche. It is known that some stem cell niches are composed of unique ECMs. Heparan sulfate proteoglycans (HSPGs) are required for restriction of gremlin stem cells in the niches in *Drosophila* (Hayashi et al., 2009). In the neural stem cell niches (e.g. the subventricular zone and subgranular zone), heparan sulfate- and chondroitin sulfate-proteoglycans regulate the proliferation and differentiation of neural stem cells in the presence of growth factors, such as epidermal growth factor and fibroblast growth factor (Akita et al., 2008). In the hair follicle stem cell niche, a hemidesmosomal transmembrane collagen, collagen XVII, plays important roles in the maintenance of both hair follicle stem cells and melanocyte stem cells (Tanimura et al., 2011). Considering these findings, our present data suggest that SOX2-positive cell clusters scattered in the parenchyma of the anterior lobe might be constructed by the production of unique ECMs that differ from the MCL.

In the present study, PS-clusters showed a lower differentiation capacity than pituispheres. In general, a sphere is formed by activating a single proliferative stem/progenitor cell using bFGF, EGF, B27- and N2-serum free supplements, which are suitable for cultivation of neural stem cells (Gritti et al., 1996; Pastrana et al., 2011; Reynolds and Weiss, 1992). Spheres are also known to lack quiescent stem cells (Pastrana et al., 2011). Indeed, although about 10% of the cells in the anterior lobe of an adult mouse are positive for SOX2, the proportion of cells showing pituisphere forming capacity is only about 0.2% of the dispersed cells from the adult mouse anterior lobe (Gremeaux et al., 2012). This clearly shows that only a small number of SOX2-positive cells are actively undergoing proliferation and differentiation. However, in the present study, we performed a differentiation assay using randomly selected PS-clusters. Considering that most of the SOX2-positive cells are in a quiescent state in the adult pituitary (Andoniadou et al., 2013), one of the reasons for these differences in the differentiation capacity between PS-clusters and pituispheres is the presence of quiescent SOX2-positive cells. In addition, SOX2-positive cells have sub-populations according to the existence of transcription factors and S100β (Higuchi et al., 2014; Yoshida et al., 2011). In the present study, using the S100β/GFP-TG rat model, we demonstrated that three subtypes of PS-clusters based on S100β-GFP signals can be isolated, and that null-GFP-clusters show different properties compared to GFP- and mixed-GFP-clusters under the culture conditions utilized (Fig. 5). Accumulating reports have demonstrated that S100β-positive cells produce several paracrine factors such as bFGF and leukemia-inhibitory factor, which are survival and/or growth factors for pituitary stem/progenitor cells (Chen et al., 2006; Denef, 2008). They also produce transforming growth factor β1, interleukin-6 and vascular endothelial growth factor of which effects for pituitary stem/progenitor cells have not yet been identified (Denef, 2008). To our knowledge, this study is the first to demonstrate the different property of SOX2-positive cells based on the existence of S100β. Our data suggest that SOX2-positive cells are heterogeneous, and a subset of these cells plays different roles such as supporting other types of SOX2-positive cells. Further studies involving comparative analysis of GFP- and null-GFP-clusters might elucidate their hierarchy in the process of differentiation, as well as the presence of niche cells.

In summary, we have demonstrated a simple method of isolating pituitary stem/progenitor cells originated from the parenchymal-niche of the anterior lobe as PS-clusters by stepwise protease dispersion. PS-clusters still maintained their stemness in conditions using Matrigel, but showed some differentiation capacity by treatment of GSK3β-inhibitor with bFGF and EGF. Therefore, further analysis of the PS-clusters might provide insight into the regulatory factors of the pituitary stem/progenitor cell niche, as well as the turnover of the hormone-producing cells.

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