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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.

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 × *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,