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Characterization of pituitary stem/progenitor cell populations in spontaneous dwarf rats

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ABSTRACT. Spontaneous dwarf rat (SDR) is a primary experimental animal model for the study of pituitary dwarfism with a point mutation in the *Gh* gene encoding growth hormone (GH). In previous studies, SDR has been reported to be associated with the GH deficiency as well as combined hormone deficiencies, the cause of which is unknown. In this study, we focused on the characteristics of pituitary stem/progenitor cell populations, which are a source of hormone-producing cells, in SDR. Immunofluorescence and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analyses confirmed the defects in GH-producing cells, the decreased number of prolactin- and thyroid-stimulating hormone-producing cells, and the increased number of adrenocorticotropic hormone- and luteinizing hormone-producing cells. Additionally, qRT-PCR analysis showed increased *Prop1* (an embryonic stem/progenitor cell marker) expression and decreased *S100b* (a putative adult stem/progenitor cell marker) expression in SDRs. In the pituitary stem/progenitor cell niche, the marginal cell layer, the proportion of SOX2/PROP1-double positive cells was higher in adult SDRs than in adult Sprague Dawley (SD) rats but that of SOX2/S100β-double positive cells was much lower. Furthermore, the number of SOX2/PROP1-double positive cells in SD rats significantly decreased with growth; however, the decrease was smaller in SDRs. In contrast, the number of SOX2/S100β-double positive cells in SD rats significantly increased with growth; however, they were few in SDRs. Thus, S100β-positive pituitary stem/progenitor cells failed to settle in pituitary dwarfism with the *Gh* gene mutation, leading to multiple hypopituitarism including GH deficiency.

KEYWORDS: pituitary gland, prophet of *Pit1*, spontaneous dwarf rat, stem/progenitor cell, S100β

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The anterior lobe of the pituitary gland consists of five types of hormone-producing cells with non-hormonal cells such as folliculo-stellate (FS) cells that are positive for S100 protein as a marker and fenestrated sinusoids (vascular endothelial cells and pericytes) that cooperate to maintain the physiological function of the gland [24, 45]. Additionally, the stemness marker *Sox2*-expressing cells, located in the pituitary stem/progenitor cell niche, called the marginal cell layer (MCL), which faces the residual lumen of Rathke's pouch, renews pituitary cells throughout life [51]. The glands of newborns already include all types of hormone-producing cell [37, 51]. However, soon after birth, the pituitary gland enters a phase of growth, resulting in a dramatic size increase [7, 42]. The approximate percentages of hormone-producing cell types in the anterior pituitary gland of adult male rats were summarized as growth hormone (GH) (45–50%), prolactin (PRL) (25%), luteinizing hormone (LH) (7–10%), thyroid-stimulating hormone (TSH) (5–9%), and adrenocorticotropic hormone (ACTH)-producing cells (3–4%) in order of greatest proportion [19]. Since adult tissues are constantly undergoing cellular replacement in response to stress, injury, and physiological demands, it is difficult to maintain tissues with only stem/progenitor cells in embryonic stages, which is consistent with the presence of adult pituitary stem/progenitor cells [10, 11, 27]. In other words, at least two or more types of stem/progenitor cells supply hormone-producing cells in adults. Several studies have reported that adult stem cells, which are qualitatively different from embryonic stem cells, maintain pituitary function after birth [3, 11, 35].

Sex-determining region Y-box 2 (SOX2) is a crucial transcription factor involved in the specification and maintenance of multiple stem cell populations in mammals. In the past 20 years, many researchers have demonstrated that SOX2-expressing cells play an essential role as stem/progenitor cells in the pituitary gland, and are able to differentiate into all types of hormone-producing cells [3, 9, 11, 16, 38]. During pituitary development, the stepwise expression of various transcription factors into SOX2-positive cells leads to the progressive differentiation into each hormone-producing cell type [51]. Although SOX2-positive

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cells are thought to have several subpopulations because of their diverse genetic characteristics [8, 9, 17, 49, 50], whether there is a difference in the roles and origins of those subpopulations is unclear [24, 35, 45].

Pituitary dwarfism, a one of hypopituitarism, is characterized by poor growth, endocrine alopecia and skin pigmentation due to a congenital decrease in GH production or release [34]. The cause is roughly classified into GH function reduction or a combined hormone deficiency in which the functions of multiple pituitary hormones including GH, are decreased. Multiple pituitary hormone deficiency (MPHD) is caused due to mutations in various transcription factors that are expressed in pituitary stem/progenitor cells and are involved in the differentiation into hormone-producing cells. The majority of genetic MPHD cases result from mutations in the transcription factors pituitary specific transcription factor 1 (PIT) or prophet of PIT1 (PROP1) [1, 40, 43, 48]. Mutations in the *Pit1* gene produce deficiencies in GH, PRL, and TSH, and pituitary hypoplasia in Snell's dwarf mice (*Pit1*dw) [6, 40, 46]. On the other hand, mutations in PROP1 cause progressive deficiencies in the same hormones as those of PIT1, with additional reductions in the levels of gonadotropic hormones, including LH and FSH, and ACTH [29, 46].

Spontaneous dwarf rats (SDRs), which were spontaneously developed from a closed colony of Sprague-Dawley (SD) rats in 1977, exhibit dwarfism with abnormal GH synthesis and release [33] due to a splicing abnormality in the *Gh* gene, a single base substitution (G to A) in the third intron [41]. Additionally, a small number of PRL- and TSH-producing cells and low reproductive function have also been reported [32]. These findings have suggested that SDR is not a model of GH-only deficiency but a model of the complex type of anterior pituitary hormone deficiency.

In this study, we focused on stem/progenitor cell populations in the pituitary gland of the pituitary dwarf model SDR. We confirmed by immunofluorescence analysis that the pituitary gland in SDRs had fewer PRL- and TSH-producing cells and more ACTH- and LH-producing cells than that in SD rats. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) showed that the expression levels of *Prop1* (an embryonic stem/progenitor cell marker) were higher in SDRs than in SD rats; however, the expression of *S100b* (a putative adult stem/progenitor cell marker) decreased. Furthermore, the proportion of SOX2/PROP1-double positive (SOX2/PROP1-positive) cells was higher but that of SOX2/S100β-double positive (SOX2/S100β-positive) cells was much lower in SDRs than in SD rats. Thus, S100β-positive pituitary stem/progenitor cells failed to settle in the pituitary gland of SDR, which may be responsible for the reduced number of *Pit1*-lineage hormone-producing cells other than GH-producing cells.

MATERIALS AND METHODS

Animals

Eight-week-old male female SD rats and SDRs were purchased from Japan SLC (Shizuoka, Japan). Food and water were provided *ad libitum*, and the rats were housed individually in a temperature-controlled room under a 12-hr light/12-hr dark cycle throughout the experiment. Adult rats of the same strain were mated, and the day when a vaginal plug was observed was designated as embryonic day 0.5. Postnatal pituitaries were surgically removed after sacrificing the young (postnatal day 15) and adult male rats (postnatal day 120). The study was approved by the Institutional Animal Care and Use Committee of Tottori University, and it was conducted in compliance with the NIH Guidelines for Animal Care and Use of Laboratory Animals.

Immunofluorescence analysis

Pituitary glands were fixed with 4% paraformaldehyde in 20 mM HEPES buffer (pH 7.5) at 4°C for 20 hr, and immersed with 30% trehalose in HEPES buffer at 4°C for 24 hr, and embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) at −80°C. Frozen sections (8-μm thick) were prepared from the coronal planes of pituitary glands. Immunofluorescence analysis was performed as previously described [14, 16, 23, 38, 49, 50] using primary and secondary antibodies listed in Table 1. Immunofluorescence was detected using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The absence of an observable nonspecific reaction was confirmed using normal rabbit, goat, or guinea pig serum. The specificity of antibodies has been referred to previous literature, as shown in Table 1. The number of SOX2- and PROP1- or S100β-positive cells together with 4,6-diamidino-2-phenylindole (DAPI)-stained cells, were counted in the MCL of the anterior lobe (n=3–5/section, respectively), and the proportion of PROP1- or S100β-positive cells among SOX2-positive cells was calculated (means ± standard deviation).

qRT-PCR

Total RNA from the anterior lobe of pituitary glands was extracted using NucleoSpin RNA Plus XS (Macherey-Nagel, Duren, Germany). cDNA synthesis and qRT-PCR were performed as previously described [16]. qRT-PCR assays were performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with gene-specific primers and SYBR Green Real-time PCR Master Mix Plus (Toyobo Co., Ltd., Osaka, Japan). The primer sequences are shown in Table 2. Each sample was assayed in triplicate in two independent experiments. Dissociation curves were generated at the end of each qRT-PCR run to ensure that a single specific product was amplified. PCRs in the absence of template cDNA served as negative controls for each primer set. qRT-PCR products were normalized to the TATA-box-binding protein (*Tbp*) and quantified. The relative gene expression was calculated by comparing the cycle times (comparative CT method) for each targeted PCR.

DNA sequencing

Rat *Prop1* and *S100b* full-length open reading frames were amplified from cDNA using PrimeSTAR MAX DNA polymerase (Takara Bio, Kusatsu, Japan) and the following primers: rat *Prop1* (NM_153627.1), 5′-ATGGAAGCTCAAAGAAGGAGC-3′

Table 1. List of antibodies used for immunofluorescence analysis

Antigen	Species	Cat. No	Identifier	References
Primary antibodies				
Rat PROP1 (1:500)	Guinea pig	-	Dr. Y. Kato (Meiji University, Kawasaki, Japan)	[49, 50]
Human SOX2 (1:400)	Goat	GT15098	Neuromics (Minneapolis, MN, USA)	[16, 49, 50]
Rat S100β (1:1,000)	Rabbit	IS504	Dako (Glostrup, Denmark)	[23]
Human ACTH (1:5,000)	Guinea pig	-	Dr. S. Tanaka (Shizuoka University, Shizuoka, Japan)	[14, 16]
Human GH (1:2,000)	Guinea pig	-		
Rat LHβ (1:5,000)	Guinea pig	-	Kindly provided by National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) through the courtesy of Dr. A. F. Parlow	[14, 16]
Rat TSHβ (1:20,000)	Guinea pig	-		
Rat PRL (:10,000)	Guinea pig	-		
Secondary antibodies				
Rabbit IgG (1:500), Cy3	Donkey	711-166-152		-
Guinea pig IgG (1:500), Cy3	Donkey	706-166-148	Jackson ImmunoResearch (West Grove, PA, USA)	-
Goat IgG (1:500), FITC	Donkey	705-096-147		-

PROP1, prophet of *Pit1*; SOX2, sex-determining region Y-box 2; ACTH, adrenocorticotropic hormone; GH, growth hormone; LHβ, luteinizing hormone beta-subunit; TSHβ, thyroid-stimulating hormone beta-subunit; PRL, prolactin.

Table 2. List of primer sets for qRT-PCR

Gene	Accession No.	Forward primer (5′→3′)	Reverse primer (5′→3′)
Pomc	NM_139326.2	AGGACCTCACCACGGAAAG	ACGTACTTCCGGGGATTTTC
Lhb	NM_012858.2	CCTGGCTGCAGAGAATGAGT	GTAGGTGCACACTGGCTGAG
Tshb	NM_013116.2	AGTGTGCCTACTGCCTGACC	GGGAAGAAACAGTTTGCCATT
Gh	NM_001034848.2	GGACCGCGTCTATGAGAAAC	GCTTGAGGATCTGCCCAATA
Prl	NM_012629.1	GCCAAAGAGATTGAGGAACAA	ATGGGAGTTGTGACCAAACC
Tbx19	NM_001394230	AGTGGAGGTGGGCAGATAATG	CTTGAGGTGGTTTCTCCTCGTTC
Nr5a1	NM_001191099	ATCTACCGCCAGGTCCAGTA	GACAGACAAACTCCTGGCGG
Gata2	NM_033442	CCACTACCTGTGCAATGCCT	AATTTGCACAACAGGTGCCC
Esr1	NM_012689.1	TGAAGGCTGCAAGGCTTTCT	GGTCTTTTCGTATCCCGCCT
Pit1	NM_013008.3	GAGAACTGGAGCAGTTTGCCAA	GACTGAATTCTGAGCCGTGGACA
Sox2	NM_001109181.1	ATTACCCGCAGCAAAATGAC	CTAGTCGGCATCACGGTTTT
Prop1	NM_153627.1	TCCTGACATCTGGGTTCGAG	GGAGTAGTGACCGCTCTTGC
S100b	NM_013191.1	ACGAGCTCTCTCACTTCCTGGA	AGTCACACTCCCCATCCCC
Tbp	NM_001004198.1	GATCAAACCCAGAATTGTTCTCC	ATGTGGTCTTCCTGAATCCC

(F) and 5′-TTAGTTCCAGGACTTTGGCG-3′ (F); rat *S100b* (NM_013191.1), 5′-AGAGGACTCCGGCGGCAAAA-3′ (F) and 5′-ATGTCTGCCACGGGGAAACG-3′ (R). The RT-PCR conditions were as follows: 35 cycles of 98°C for 10 sec, 55°C for 5 sec, and 72°C for 10 sec, and the amplified products were subjected to DNA sequencing using a BigDye Terminator version 3.1 and ABI3130 sequencer (Applied Biosystems, Carlsbad, CA, USA). Dot plot images were created using BLAST from the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

qRT-PCR data were analyzed using Student's and Welch's *t*-tests for comparisons between two groups, and immunofluorescence data were analyzed using two-way analysis of variance followed by Tukey's test for multiple comparisons among the four populations.

RESULTS

Differences in the appearance and pituitary glands of SD rats and SDRs

The weight of adult male SDRs (107 ± 14 g, n=5) was approximately one-fifth of that of adult SD rats (520 ± 18 g, n=5), and a dramatic difference was observed in the appearance of both rats (Fig. 1A). Additionally, the pituitary gland of SDRs (5.3 ± 0.7 mg) was smaller than that of SD rats (16.3 ± 0.9 mg). Next, we performed immunofluorescence analysis for five types of anterior pituitary hormones, including GH, PRL, TSHβ, ACTH, and LHβ, to confirm the localization of immune-positive cells in pituitary glands on adult rats (Fig. 1B). GH immune-positive cells were not observed in SDRs. PRL- and TSHβ-positive cells were fewer in SDRs than in SD rats, but ACTH- and LHβ-positive cells more in SDRs. Furthermore, the qRT-PCR data corresponded to the results of immunofluorescence analysis (Fig. 1C).

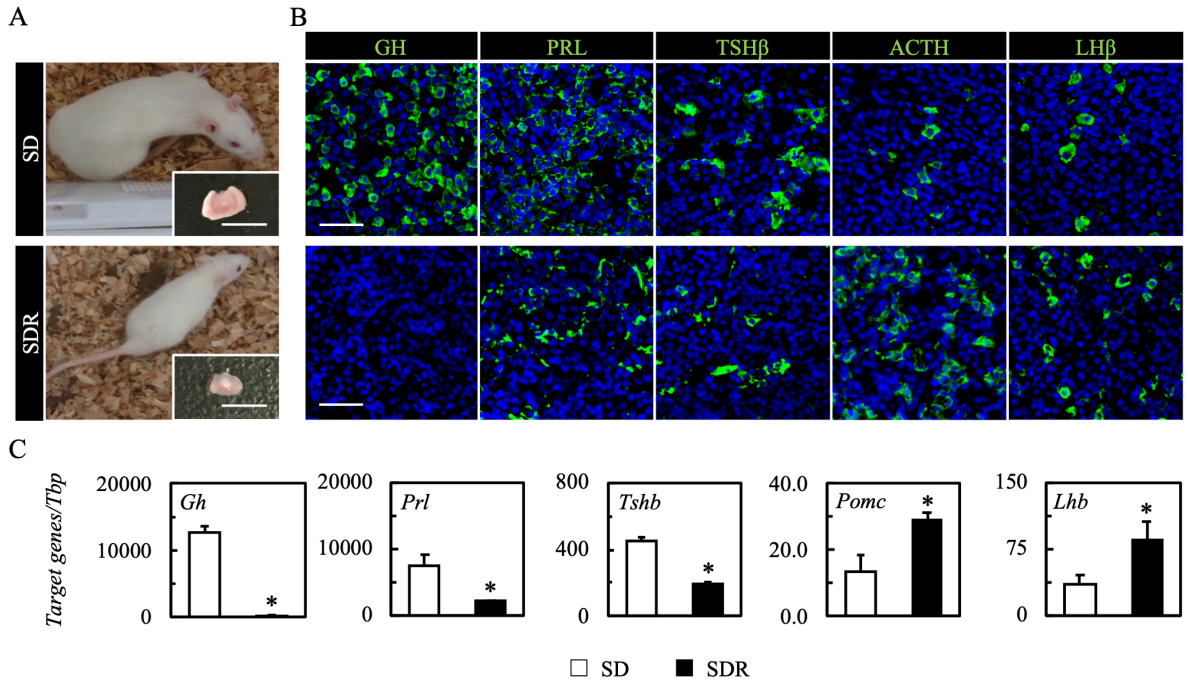

Fig. 1. Comparisons of the appearance, anterior hormonal genes and immuno-positive cells in the pituitary glands of adult Sprague-Dawley (SD) rats and spontaneous dwarf rats (SDRs). (**A**) Images of adult SD rats and SDRs are shown, and dissected pituitary glands are enlarged in lower right panels. (**B**) Immunofluorescence analysis was performed with specific antibodies, and cells positive for each hormone [growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone β (TSHβ), adrenocorticotropic hormone (ACTH), and luteinizing hormone β (LHβ)] were visualized using Alexa Fluor 488 (*green*). Merged images with 4,6-diamidino-2-phenylindole (DAPI, *blue*) are shown. Scale bars=5 mm (A) and 50 μm (B). (**C**) Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed to estimate the mRNA levels of each anterior hormonal gene (*Gh*, *Prl*, *Tshb*, *Pomc*, and *Lhb*). Data were calculated using the comparative Ct method to estimate the relative amount against the transcripts of TATA-box-binding protein (*Tbp*) gene used as an internal standard. Data are presented as means ± standard deviation (n=3, in each group). Asterisks indicate a statistically significant difference between the two groups (P<0.01).

Gene expression of pituitary stem/progenitor cell and commitment cell markers in SD rats and SDRs

qRT-PCR analysis showed that the expression levels of *Tbx19* (a transcription factor for differentiation into ACTH-producing cells) and *Nr5a1* (a transcription factor for differentiation into LH-producing cells) in SDRs were significantly higher than those in SD rats (Fig. 2A). On the other hand, differences in the expression levels of *Gata2* (a transcription factor for differentiation into TSH- and LH-producing cells), *Esr1* (a transcription factor for differentiation into PRL-producing cells), and *Pit1* (a transcription factor for differentiation into GH-, TSH-, and PRL-producing cells) were not observed between SD rats and SDRs. Further, no difference was observed in the expression level of *Sox2* in SD rats and SDRs. On the other hand, the expression level of *Prop1* was higher in SDRs than in SD rats; however, *S100b* expression was lower. Finally, we compared the coding sequences of *Prop1* and *S100b* in SD rats and SDRs using a dot plot, and found no difference between the two groups (Fig. 2B).

Characterization of SOX2-positive pituitary stem/progenitor cells in SD rats and SDRs

To clarify whether the characteristics of pituitary stem/progenitor cells in SDRs differ from that of SD rats, we performed immunofluorescence analysis for SOX2 and PROP1 or S100β (Figs. 3 and 4). SOX2-positive cells were widely distributed throughout pituitary glands of SD rats and SDRs. Focusing on the pituitary stem/progenitor cell niche, the MCL, the number of SOX2-positive cells in SDRs was lower than that in SD rats (young SD rats: 278 ± 42 cells *vs.* SDRs: 181 ± 7 cells, P<0.01; adult SD rats: 247 ± 20 cells *vs.* SDRs: 175 ± 12 cells, P<0.01). Additionally, in the MCL of the pituitary gland, the percentages of SOX2-signals (SOX2+/DAPI) were slightly lower in SDRs than in SD rats (young SD rats: 76.7 ± 4.7% *vs.* SDRs: 68.0 ± 3.5%, P<0.01; adult SD rats: 72.2 ± 9.2% *vs.* SDRs: 59.4 ± 4.7%, P<0.01). Further, the proportion of PROP1-positive cells among SOX2-positive cells was higher in SDRs than in SD rats (young SD rats: 32.6 ± 2.5% *vs.* SDRs: 58.6 ± 5.6%, P<0.01; adult SD rats: 6.0 ± 0.8% *vs.* SDRs: 27.9 ± 4.8%, P<0.01).

In contrast, S100β-positive cells were present in the anterior lobe of young SD rats; however, few S100β-positive cells were present in that of young GH mutant SDR. In addition, the percentages of S100β-positive cells (S100β+/DAPI) in the anterior lobe side of the MCL were 12.5 ± 2.9% for young SD rats and 0.1 ± 0.2% for young SDRs (P<0.01). In adult rats, the difference was more pronounced (adult SD rats: 42.3 ± 3.9% *vs.* SDRs: 2.7 ± 0.9%, P<0.01). Moreover, the proportion of S100β-positive cells among SOX2-positive cells was lower in SDRs than in SD rats (young SD rats: 15.9 ± 2.6% *vs.* SDRs: 0.2 ± 0.3%, P<0.01; adult SD rats: 59.6 ± 7.3% *vs.* SDRs: 4.9 ± 1.7%, P<0.01).

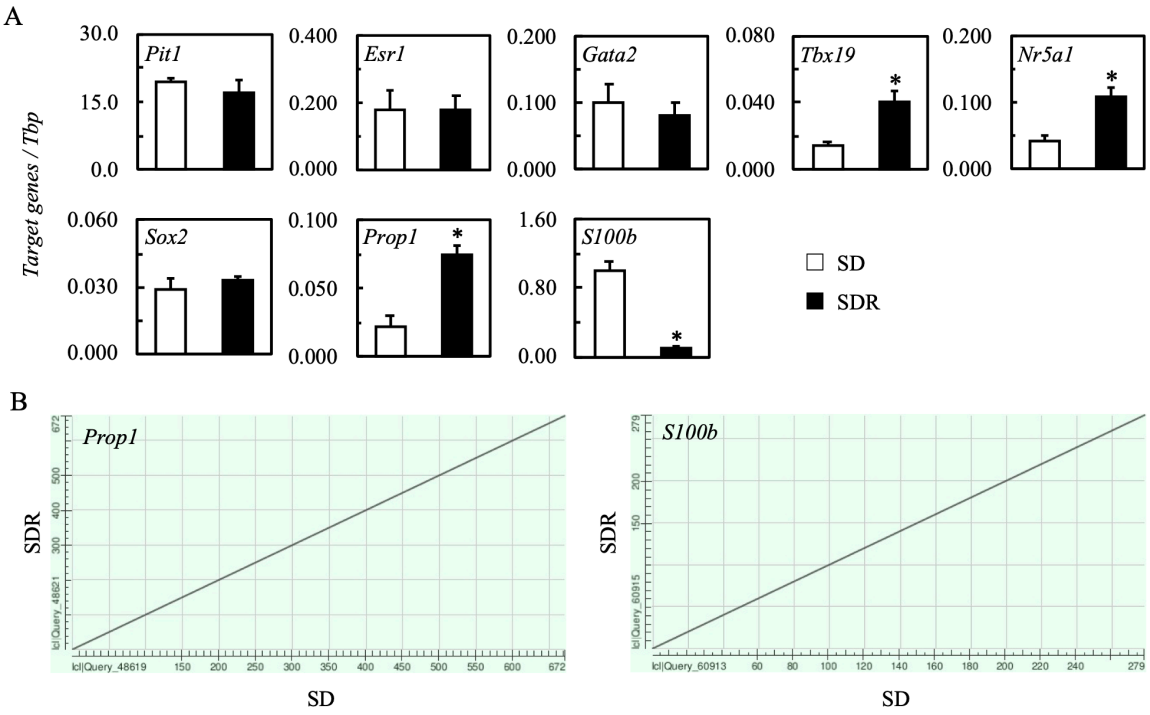

Fig. 2. Expression levels of markers for pituitary stem/progenitor, commitment and terminally differentiated cells in the pituitary glands of adult Sprague-Dawley (SD) rats and spontaneous dwarf rats (SDRs) and DNA sequencing. (**A**) Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed to estimate the mRNA levels of *Pit1*, *Esr1*, *Gata2*, *Tbx19*, *Nr5a1*, *Sox2*, *Prop1*, and *S100b*. Data were calculated using the comparative Ct method to estimate the relative amount against the transcripts of the TATA-box-binding protein (*Tbp*) gene used as an internal standard. Data are presented as means ± standard deviation (n=3, in each group). Asterisks indicate a statistically significant difference between the two groups ($P<0.01$). (**B**) Rat *Prop1* and *S100b* full-length open reading frames were amplified from complementary DNA, which was synthesized from the anterior lobe of pituitary glands in SD rats and SDRs, and they were subjected to DNA sequencing using a BigDye Terminator version 3.1 and ABI3130 sequencer. Dot plot images with nucleotide sequences between SD rats and SDRs are shown in left (*Prop1*) and right panels (*S100b*).

DISCUSSION

The pituitary dwarfism model SDR not only causes growth retardation associated with GH deficiency but also possibly leads to a complex type of anterior pituitary hormone deficiency; however, the details are unknown. The fact that characteristics of SOX2-positive cell populations, located in the MCL, were different between SD rats and SDRs indicated that the defect of functional GH-producing cells affected the quality of stem/progenitor cells in the pituitary gland. These results suggested that each subpopulation of SOX2-positive pituitary stem/progenitor cells had a different cell fate.

Several transcription factors for cell lineage commitments are spatiotemporally expressed in the process of cell differentiation, leading to the transcriptional activation of pituitary hormone-coding genes [51]. *Tbx19* gene encoding T-box transcription factor 19 is a well-known transcription factor that upregulates the expression level of the *Pomc* gene by binding to a promoter region, and is only present in the two pituitary *Pomc*-expressing lineages, the corticotrophs (ACTH-producing cells in the anterior lobe) and melanotrophs (alpha-melanocyte-stimulating hormone-producing cells in the intermediate lobe) [25]. *Nr5a1* gene encoding steroidogenic factor 1 is a transcription factor found in adrenal and gonadal development, and a knockout of the *Nr5a1* gene in the pituitary gland is selectively deficient in gonadotropic gene expression (*Lhb* and *Fshb*) [21]. In this study, the expression levels of *Tbx19*, *Nr5a1*, *Pomc*, and *Lhb* were higher in SDRs than that in SD rats, and higher densities of ACTH- and LHβ-positive cells were found in SDRs than that in SD rats. Recently, we revealed that the percentage of ACTH- and LH-producing cells decreases with the dramatic expansion of the pituitary gland during the postnatal period (the so-called postnatal growth wave), while the percentage of GH-, PRL-, and TSH-producing cells increases [13]. The high densities of ACTH- and LH-producing cells in the adult SDR pituitary gland may be attributed to the lack of the expansion of the pituitary gland with growth. A possible explanation for this is that the number of *Pit1*-lineage hormone-producing cells did not increase significantly.

Pit-1 activates transcription by binding to the promoter regions of genes such as *Gh*, *Prl,* and *Tshb* [12, 20]. Therefore, in *Pit-1* mutant mice, the reduced DNA binding capacity of PIT1 causes GH, PRL, and TSHβ defects [46]. *Esr1* gene encoding estrogen receptor alpha forms a complex with estrogen, followed by an initiation of *Prl* gene expression and a stimulation of PRL secretion [36]. In addition, *Gata*2 gene encoding GATA binding protein 2 governs the cell-specific expression of the *Tshb* gene through

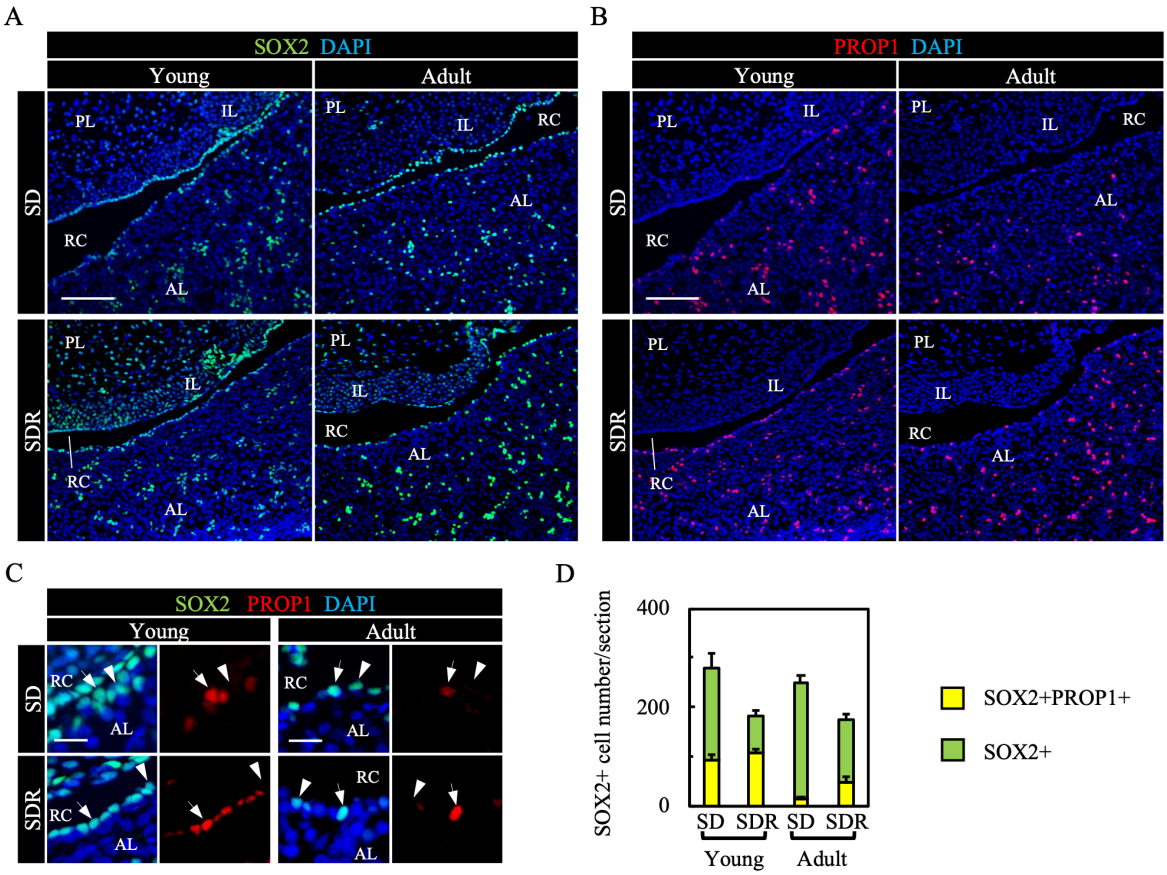

Fig. 3. Immunofluorescence analysis of sex-determining region Y box 2 (SOX2) and prophet of *Pit1* (PROP1) in the pituitary gland of Sprague-Dawley (SD) rats and spontaneous dwarf rats (SDRs). (**A–C**) Double-immunostaining for SOX2 (**A**, *green*) and PROP1 (**B**, *red*) were performed in the pituitary glands of young (postnatal day 15) and adult (postnatal day 120) rats, and images merged with 4,6-diamidino-2-phenylindole (DAPI, *blue*) are shown. Enlarged images of the pituitary stem/progenitor cell niche, the marginal cell layer (MCL), of the anterior lobe in pituitary glands are shown in **C**. Arrows and arrowheads show SOX2/PROP1 double-positive (SOX2+PROP1+) and SOX2 single-positive (SOX2+) cells, respectively. (**D**) Numbers of SOX2+PROP1+ (yellow bars) and SOX2+ (green bars) in the MCL (n=5/section, respectively). Scale bars=200 µm (A, B), 20 µm (C). AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe; RC, Rathke's cleft.

direct interaction with PIT1 [12]. In the present study, through an analysis of gene expression, no difference was observed in the expression levels of *Pit1*, *Esr1*, and *Gata2* genes in SD rats and SDRs. Nevertheless, densities of PRL and TSHβ immune-positive cells were low in addition to showing reduced expression levels of *Prl* and *Tshb* genes. These results suggested the existence of a differentiation pathway independent of *Pit1*. In fact, we recently reported the presence of PIT1 non-expressing GH, PRL, and TSH-producing cells in mice [15]. Based on these findings, we suggested that the SDR pituitary gland lacks the stem/progenitor cell population, which differentiate into the *Pit1*-lineage hormone-producing cells (PRL, TSH, and GH).

Several studies using transgenic mice deficient in GH-producing cells have demonstrated that the secondary reduction in *Pit1*-lineage hormone-producing cells, except for GH, is due to a loss of functional GH-producing cells associated with defective GH secretion (defective in both synthesis and release) [4, 5]. However, mice that can synthesize GH but lack GH-releasing hormone (GHRH) or its receptor (e.g., GHRH knockout and *little* mice) are reported to have unchanged percentages of PRL- and TSH-producing cells [2, 26]. To prevent the secondary reduction in PRL- and TSH-producing cells noted in the SDR pituitary gland, it may be necessary to rescue the GH-producing cells, with no abnormality in the GH synthesis, rather than the circulating GH protein itself. Further studies are needed to determine the cause of secondary decreased PRL- and TSH-producing cells; a transplantation of GH-producing cells and repair of the *Gh* mutation by gene editing may elucidate these observations.

The present study revealed that the proportion of PROP1-positive cells among SOX2-positive cells in SD rats decreased markedly with growth; however, the decrease was smaller in SDRs. This suggests that, in SDRs with a GH gene mutation, a part of PROP1-positive stem/progenitor cells that are present in the embryonic stages (Rathke's pouch), do not differentiate and remain in the MCL. In fact, almost all the cells in Rathke's pouch are SOX2/PROP1-positive cells, and the percentage gradually decreases during the course of development [49, 50]. In contrast, we found that the SOX2-positive pituitary stem/progenitor cell population positive for S100β was almost non-existent in the MCL of SDRs. These results suggest that the deficiency of functional GH-producing cells inhibits the settlement of S100β-positive adult pituitary stem/progenitor cells to the MCL. S100β-positive cells are

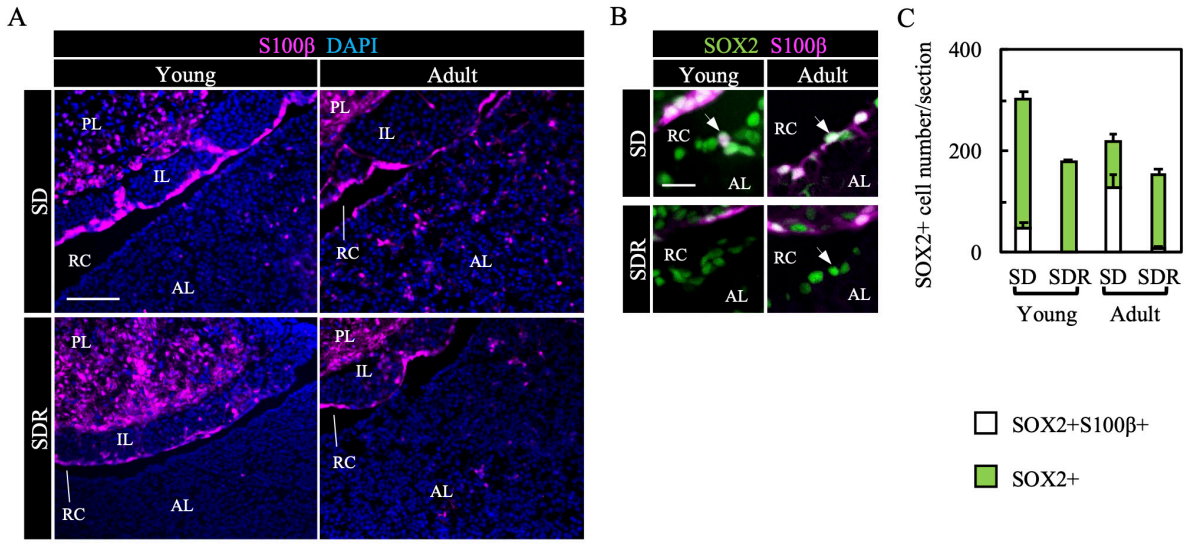

Fig. 4. Immunofluorescence analysis of sex-determining region Y box 2 (SOX2) and S100β in the pituitary gland of Sprague-Dawley (SD) rats and spontaneous dwarf rats (SDRs). (**A**, **B**) Double-immunostaining for SOX2 (*green*) and S100β (*purple*) were performed in the pituitary glands of young (postnatal day 15) and adult (postnatal day 120) rats, and images merged with 4,6-diamidino-2-phenylindole (DAPI, *blue*) are shown. Enlarged images of the pituitary stem/progenitor cell niche, the marginal cell layer (MCL), of the anterior lobe of the pituitary glands are shown in B. Arrows show SOX2/S100β double-positive cells (SOX2+S100β+). (**C**) Numbers of SOX2+S100β+ (white bars) and SOX2+ (green bars) in the MCL (n=3/section, respectively). Scale bars=200 μm (A), 20 μm (B). AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe; RC, Rathke's cleft.

not present in the anterior lobe during fetal life and immediately after birth; however, they appear in the anterior lobe at postnatal days 3 to 10 [39, 44]. Additionally, we have demonstrated that SOX2/S100β-positive cells showed a high proliferation activity and differentiation potential into hormone-producing cells [14]. Based on these findings, S100β/SOX2-positive cells were considered to be putative adult pituitary stem/progenitor cells that contributed to the maintenance of post-growth pituitary function through the differentiation into *Pit1*-lineage hormonal cells.

Nogami and Takeuchi reported that cell populations positive for six types of anterior pituitary hormone antibodies in male SDR and SD rat pituitary glands are approximately 45.0% and 81.3%, respectively [31]. These findings imply that, in the SDR pituitary gland, the percentage of non-hormonal cells increased with the loss of functional GH-producing cells. The FS cells, one of non-hormonal cells, in the SDR pituitary gland display the same ultrastructural characteristics as those in the SD rat pituitary gland [30]. However, in this study, the presence of cells positive for S100β, known as the marker for FS cells [28], was rarely observed in the anterior lobe of the SDR pituitary gland. This result supports the findings of previous studies indicating that S100β-positive cells are dramatically fewer in SDR than in SD rat [47]. The findings further demonstrated that S100α-positive cells are numerous in the pituitary gland of both SD rat and SDR [47]. The S100 protein consists of a dimer of two subunits, α (S100α) and β (S100β), and is present in three forms (αα, aβ, and ββ) [22]. Taken together, we speculate that subpopulations of pituitary FS cells in which S100β is predominantly expressed may function as pituitary stem/progenitor cells.

In summary, we revealed the characteristics of SOX2-positive stem/progenitor cell populations in the pituitary gland of SDRs, especially in the MCL. It has been reported that S100β-positive cells migrate from the intermediate lobe to the anterior lobe [17, 18]. The results of this study suggested that GH-producing cells not only secrete hormones to stimulate growth and work for lipolysis but were also involved in attracting adult pituitary stem/progenitor cells to the MCL of the anterior lobe. Further studies are needed to determine whether GH itself or the triggers produced by GH-producing cells contribute to the migration of S100β-positive cells.

CONFLICT OF INTEREST. The authors declare no competing interests.

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