The astroglial and stem cell functions of adult rat folliculostellate cells

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Abstract

The mammalian pituitary gland is a complex organ consisting of hormone-producing cells, anterior lobe folliculostellate cells (FSCs), posterior lobe pituicytes, vascular pericytes and endothelial cells, and Sox2-expressing stem cells. We present single-cell RNA sequencing and immunohistofluorescence analyses of pituitary cells of adult female rats with a focus on the transcriptomic profiles of nonhormonal cell types. Samples obtained from whole pituitaries and separated anterior and posterior lobe cells contained all expected pituitary resident cell types and lobe-specific vascular cell subpopulations. FSCs and pituicytes expressed S100B, ALDOC, EAAT1, ALDH1A1, and VIM genes and proteins, as well as other astroglial marker genes, some common and some cell type-specific. We also found that the SOX2 gene and protein were expressed in ~15% of pituitary cells, including FSCs, pituicytes, and a fraction of hormone-producing cells, arguing against its stem cell specificity. FSCs comprised two Sox2-expressing subclusters; FS1 contained more cells but lower genetic diversity, while FS2 contained proliferative cells, shared genes with hormone-producing cells, and expressed genes consistent with stem cell niche formation, regulation of cell proliferation and stem cell pluripotency, including the Hippo and Wnt pathways. FS1 cells were randomly distributed in the anterior and intermediate lobes, while FS2 cells were localized exclusively in the marginal zone between the anterior and intermediate lobes. These data indicate the identity of the FSCs as anterior pituitary-specific astroglia, with FS1 cells representing differentiated cells equipped for classical FSC roles and FS2 cells exhibiting additional stem cell-like features.

KEYWORDS

astrocyte marker genes, endothelial cells, folliculostellate cells, hormone-producing cells, pericytes, pituicytes, pituitary gland, pituitary stem/progenitor cells, Sox2, stem cell marker genes

1 INTRODUCTION

The pituitary gland consists of two parts: the anterior pituitary (or adenohypophysis) composed of the anterior and intermediate lobes, and the posterior pituitary (or neurohypophysis) composed of the posterior lobe and the pituitary stalk or infundibulum, which connects the posterior lobe to the hypothalamus. The gland contains three groups of resident cells: hormone producing corticotrophs,
gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs located in the anterior lobe, and melanotrophs located in the intermediate lobe; nonhormonal folliculostellate cells (FSCs) in the anterior pituitary, pituicytes in the posterior pituitary, and endothelial cells (ECs) and pericytes from the vascular networks of the anterior and posterior pituitary; and Sox2-expressing pituitary stem/progenitor cells (PSCs) with capacity to generate all hormone-producing cells (HPCs) and probably FSCs. Hormone-producing cells are relatively well characterized (Stojilkovic et al., 2010). Initial single-cell RNA sequencing (scRNAseq) studies were helpful in the global characterization of HPCs in male mice (Cheung et al., 2018), male and female rats (Fletcher et al., 2019), and human fetal pituitaries (Zhang et al., 2020). In contrast, nonhormonal cell types and stem cells require further characterization, including the need for additional scRNAseq studies.

Based on histological and physiological studies, pituicytes and FSCs are substantially characterized as heterogeneous cell types (Allaerts et al., 1996; Chen et al., 2005; Fauquier et al., 2008; Horiguchi et al., 2010a; Le Tissier et al., 2017). These cells are defined as posterior pituitary astrocytes (Verkhratsky & Nedergaard, 2018) with genetic and functional profiles similar to tanyocytes (Chen et al., 2020; Clasadonte & Prevot, 2018). These cells are continuously replaced by proliferation and differentiation of stem-like cells (Virard et al., 2006). Sox2 appears to be a gene of interest for defining pituicyte-generating cells (Pak et al., 2014). The anterior lobe FSCs were first described almost 70 years ago (Rinehart & Farquhar, 1953) and characterized as stellate cells forming gap-junction coupled networks (Fauquier et al., 2001; Vila-Porcile, 1972). Significant progress in understanding their structure, diverse functions, and genetic profiles has been achieved, aided by the development of an S100B-GFP transgenic rat model (Devnath & Inoue, 2008; Itakura et al., 2007; Kato et al., 2021). FSCs have been reported to share both morphological and genetic features with brain astrocytes (Osuna et al., 2012), including long cytoplasmic processes and S100B protein expression (Nakajima et al., 1980). FSCs are thought to act as stem cells in the anterior pituitary gland (Horvath & Kovacs, 2002; Inoue et al., 2002; Vankelecom, 2007). However, the identity of FSCs as pituitary astroglia and their potential to act as stem cells have not yet been determined.

PSCs have been the subject of recent intensive investigation (Chen et al., 2005; Fauquier et al., 2008), with implications for understanding postnatal gland regeneration and remodeling. However, PSC research faces at least three uncertainties: their location in the pituitary gland, their transcriptome profile, and the origin of these cells. They are thought to reside in two types of stem cell niches: the marginal layer of cells lining Rathke’s cleft between the anterior and intermediate lobes and in dense clusters dispersed throughout the gland parenchyma (Yoshida et al., 2009). PSCs have also been reported to express other stem/progenitor genes known to contribute to embryonic development of pituitary cells, such as Cdh1, Cd9, Lhx3, Prop1, Prpx1, Vim, Sox9, and S100b (Gleberman et al., 2008; Horiguchi et al., 2018; Rizotto, 2010; Vankelecom & Chen, 2014). Sox2-positive cells have been detected in pituitary cells by scRNAseq studies, but contradictory conclusions have been drawn about the cell types expressing this gene. We and others reported that Sox2-expressing cells in the anterior lobe expressed genes consistent with FSC identity (Fletcher et al., 2019; Vennekens et al., 2021), in agreement with immunohistofluorescence studies (Kato et al., 2021). Others have used Sox2 expression alone to classify cells as stem cells without identifying FSCs (Cheung et al., 2018; Cheung & Camper, 2020; Lopez et al., 2021; Mayran et al., 2019; Zhang et al., 2020), effectively renaming FSCs as stem cells (Le Tissier & Molland, 2021). To date, a systematic investigation of the transcriptomic profiles of Sox2-expressing cells in relation to FSCs, pituicytes, HPCs, and vascular cells has not yet been reported (Le Tissier & Molland, 2021).

In this study, we combined scRNAseq of cells from freshly dissociated pituitary cells and immunohistofluorescence analysis to study the transcriptome profiles of FSCs, pituicytes, and vascular cells, and to evaluate the expression of astroglial and stem cell marker genes and proteins in pituitaries from adult randomly cycling female rats. As expected, we observed all resident pituitary cell types, including lobe-specific clusters of vascular cells. We compared the transcriptomic profiles of pituicytes, vascular cells, and FSCs, and we show that FSCs, like pituicytes, have significant enrichment of known astroglial marker genes and proteins, supporting their classification as anterior pituitary astroglia. We also show that the SOX2 gene and protein are well expressed among all FSCs and pituicytes, indicating its insufficiency as a sole PSC-specific marker gene. We therefore further characterized the gene expression profiles and heterogeneity of FSCs. Two FSC subpopulations were identified; the larger of the two expressed genes typical for differentiated FSCs, and the smaller one expressing genes indicative of their roles as postnatal PSCs. Immunohistofluorescence confirmed that specific markers of the latter cells were expressed in cells in the marginal zone between the anterior and intermediate lobes.

2 | MATERIALS AND METHODS

2.1 | Animals

Sprague Dawley rats were obtained from Taconic Farms (Germantown, NY) and housed for two weeks under constant temperature and humidity conditions, with light on between 06:00 and 20:00 h and water and food ad libitum. Experiments were performed with 75-day-old random cycling females. After CO2 euthanasia, decapitation and removal of the brain, pituitaries were collected and used for cell dispersion or RNA extraction as described below. For immunohistofluorescence analysis, rats were anesthetized with 3% isoflurane and then transcardially perfused with 0.1 M phosphate buffer saline (PBS) followed with 4% formaldehyde. The heads were postfixed overnight. Pituitaries were removed and cryoprotected in graded sucrose-PBS solution (10% for 4 h, 20% and 30% until the tissue sank) at 4°C. Pituitaries were frozen in dry ice in Tissue-tek O.C.T Compound (Sakura; Torrance, CA). Tissue sections (14 μm) were mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and kept at −80°C. All experimental procedures were approved by the National
Institute of Child Health and Human Development, Animal Care and Use Committee (Animal Protocol 19-041).

2.2 Dispersal of pituitary cells

After decapitation, the dorsal cranial bones were dissected, the whole brain was lifted and removed to expose the pituitary gland fully. A membrane covering gland was removed, the whole pituitary was lifted, or the top part of pituitary containing posterior lobes was lifted initially, followed by collection of the anterior lobes. In this procedure, the whole intermediate lobe was removed together with posterior lobe as documented by scRNAseq data showing that all melanotrophs, the resident cells of the intermediate lobe, were found in the preparation from posterior lobe. A small fraction of anterior pituitary cells was also detected in the posterior pituitary preparation. In total 30 whole pituitaries, 30 posterior/intermediate lobes, and 30 anterior lobes were quickly collected and kept in ice-cold 199 Hanks media. The tissue was then washed with ice-cold PBS supplemented with 0.2% bovine serum albumin fraction V (MP Biomedical, Solon, OH) and 1.26 mM CaCl$_2$ (Quality Biological, Gaithersburg, MD). All collected tissue was chopped into 0.6 x 0.6 mm pieces and incubated in a water bath shaker for 25 min at 50 rpm, in the presence of a trypsin solution (4 mg/ml; Sigma, St Louis, MO), which was then replaced with a solution containing 4 mg/ml trypsin inhibitor (Sigma) for 5 min. The latter was removed, and 2 mM EDTA (Corning, Manassas, VA) solution was added over 5 min and then replaced with a 1 mM EDTA solution over 10 min with constant shaking. The volume of media was proportional to the weight of the tissue. After these steps, tissue was mechanically dispersed/dissociated by gently drawing the pituitary fragments in and out of a siliconized Pasteur pipette. When dispersion was completed, cells were transferred to a plastic tube, centrifuged, the supernatant was discarded and cells were resuspended, counted, and immediately used for scRNAseq and RNA isolation, or cultured overnight for other experiments.

2.3 Quantitative RT-PCR

RNA was extracted from individual anterior and posterior pituitary glands, and cultured anterior pituitary cells 30 min after cell dispersion, using a RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed with a Transcripter First Strand cDNA Synthesis Kit (Roche Applied Sciences, Indianapolis, IN). Quantitative RT-PCR was performed using Applied Biosystems pre-designed TaqMan Gene Expression Assays for rats and TaqMan^® Fast Advanced Master Mix. PCR was performed in the QuantStudio 6 Flex Real-Time System (Applied Biosystems, Waltham, MA). Target gene expression levels were determined by the comparative 2^-delta C(T) quantification method using Gapdh as the reference gene, which was previously established to be a suitable reference gene for the rat anterior pituitary tissue (Janjic et al., 2019). Applied Biosystems predesigned TaqMan Gene Expression Assays were used: Aldh1a1 (Rn00755484_m1), Aldh1l1 (Rn00674034_m1), Aap4 (Rn01401327_s1), Edn3 (Rn01755284_m1), Fbn1 (Rn01514386_s1), Gapdh (Rn01426622_g1), Gmap (Rn01253033_m1), Gstm2 (Rn00598597_m1), Maob (Rn00566203_m1), Nes (Rn01455599_g1), Pck1 (Rn00657566_m1), S100a1 (Rn01458753_m1), S100a6 (Rn00821474_g1), S100a10 (Rn06378613_s1), S100a11 (Rn01409258_g1), S100a13 (Rn01769833_m1), S100c6 (Rn01458849_g1), S100b (Rn04219408_m1), Slc1a2 (Rn00691548_m1), Slc103 (Rn01402419_g1), Sox2 (Rn01286286_g1), Sox9 (Rn01751070_m1), Sult1a1 (Rn01510633_m1), and Vim (Rn00667825_m1).

2.4 Immunohistochemistry

The following antibodies (with RRID) were used: mouse anti-S100B (Novus Biologicals, Littleton, CO; AB_2184561); rabbit anti-S100B (DAKO; AB_10013383); rabbit anti-S100A1 (GeneTex, Irvine, CA; AB_372948); rabbit anti-ALDH1A1 (GeneTex, Irvine, CA; AB_11164467); rabbit anti-SOX2 (GeneTex, Irvine, CA; AB_2038021); mouse anti-ALDO (generous gift from Prof. Richard Hawkes, Department of Cell Biology & Anatomy and Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, Alberta, Canada; AB_2315621); mouse anti-EEA1/SLC1A3 (generous gift from Dr. Susan Wray, Miltenyi Biotec, Bergisch Gladbach, Germany; AB_10829302), mouse anti-vimentin (generous gift from Dr. Susan Wray, Boehringer, Ingelheim, Germany; AB_2921695); rabbit anti-FAM46B/TENT5B (Proteintech, Rosemont, IL; AB_2879218); mouse anti-KRT17 (Santa Cruz Biotechnology, Dallas, TX; AB_2893006); rabbit anti-KRT17 (generous gift from Prof. Pierre A. Coulombe, Department of Cell and Developmental Biology, University of Michigan Medical School; AB_2921696). Secondary antibodies used: Alexa Fluor 568 goat anti-mouse, Alexa Fluor 568 donkey anti-rabbit, Alexa Fluor 488 goat anti-mouse or Alexa Fluor 488 goat anti-rabbit (Invitrogen, Carlsbad, CA).

For double immunostaining protocol, pituitary sections were kept at room temperature (RT) for 2 h. After washing twice in PBS for 5 min, antigen retrieval was performed. Sections were put in universal heat-induced epitope retrieval (Abcam, UK) for 30 min on 80°C in a water bath, which was followed by cooling at RT for 30 min. From this point, every step of immunostaining protocol was followed by washing sections three times in PBS. Sections were incubated overnight at 4°C with primary rabbit anti-S100A1 (1:200), rabbit anti-ALDH1A1 (1:200), mouse anti-ALDO (1:200), mouse anti-EEA1/SLC1A3 (1:200), mouse anti-vimentin (1:4), rabbit anti-SOX2 (1:200), rabbit anti-FAM46B/TENT5B (1:50), rabbit anti-KRT17 (1:2000), or mouse anti-KRT17 (1:2000), followed by application of the corresponding secondary antibodies (Alexa Fluor 568 goat anti-mouse or Alexa Fluor 568 donkey anti-rabbit, Alexa Fluor 488 donkey anti-mouse or Alexa Fluor 488 goat anti-rabbit (Invitrogen, Carlsbad, CA).
incubated in PBS containing 5% BSA for 1 h at RT. To confirm the specificity of immunostaining, control sections were subjected to the same procedure, with the omission of primary antibodies (Figure S1). All antibodies were diluted in staining PBS solution containing 0.5% BSA and 0.5% Triton X-100. Sections were mounted with Fluoromount-G, with 4',6-diamidino-2- phenylindole (DAPI; Invitrogen, Carlsbad, CA). All images were acquired on an inverted confocal laser-scanning microscope (LSM 780; Carl Zeiss GmbH, Jena, Germany), using a 63x oil objective.

2.5 | Single-cell RNA sequencing

2.5.1 | Library preparation, sequencing, and transcript counting

Freshly dispersed cells from two types of cell preparations were used, one whole-pituitary and the other with anterior and posterior pituitaries separated. The whole pituitary sample was run in duplicates and the anterior and posterior samples were each loaded into separate lanes, for a total of four lanes on the 10X Genomics Chromium controller loaded at 10,000 cells per lane according to manufacturer’s instructions. Resulting libraries were sequenced on an Illumina HiSeq 2500, and the CellRanger pipeline (10X Genomics) was used for mapping reads and counting transcripts. A custom reference genome was used as previously described (Fletcher et al., 2019), based on the Ensembl Rnor6.0 genome, with each gene extended up to 6 kb downstream or until the next feature was encountered. The four samples were aggregated with CellRanger aggr, resulting in a total of 32,455 droplets, with a mean of 20,950 reads, a median of 1803 unique genes, and a median of 5693 UMI per droplet. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE184319 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184319).

2.5.2 | Dimensionality reduction, visualization, and clustering

For visualization and reporting of expression levels, transcript counts were normalized to total cell library size and log transformed with pseudocount of 1. Dimensionality reduction was performed in the R package Seurat v4.1 (Hao et al., 2021) using SCTransform normalization of raw counts with 2000 variable genes (glmGamPoi method), followed with PCA, retaining 50 components. Distances between cells in the resulting PC space were measured using correlation distance and used for the subsequent k-nearest neighbor (kNN) identification (knnsearch in MATLAB, R2021b; The MathWorks, Inc., Natick, MA) and UMAP embedding (umap-learn version 0.5.1) (McInnes et al., 2018). Unsupervised clustering of the graph generated by UMAP was performed using the Leiden algorithm (leidenalg version 0.8.1) (Traag et al., 2019). Violin plots were made using ViolinPlot (Bechtold, 2016), and all other plots were generated using MATLAB.

2.5.3 | Cell filtering

The transcriptomes of cell types of the pituitary are heterogeneous, so cell quality control was performed in a cluster-based manner (Figure S2). First, unsupervised clustering was performed (resolution = 1) and the distributions of cell quality metrics were inspected per cluster. Clusters composed entirely of droplets with low number of genes and high fraction of counts attributed to mitochondrial transcripts (fracMT) were identified and removed. In the remaining clusters, thresholds for high fracMT and low total gene count were defined for each cluster as the median ± 3 median absolute deviations, respectively. A global lower limit of 750 genes and upper limit of 30% mitochondrial fraction was used in cases where the adaptive thresholds exceeded these values. This procedure removed 14,828 droplets. Also removed were a small cluster of erythrocytes (164 cells), and two small clusters of unidentified cells from the posterior lobe sample, which we deemed too few to warrant further analysis (72 cells total).

2.5.4 | Cell classification and identification of ambiguous cells

To address the possibility of multiplet cells or putative multihormonal cells, we used a cell-wise classification strategy based on cell type marker genes. This approach allowed for the identification and analysis of cells expressing signatures of more than one cell type. Cell clusters remaining after outlier removal could be readily identified for each expected pituitary cell type based on canonical marker genes for expected pituitary cell types. We used this initial classification to find genes with dominant expression in each cell type cluster, focusing on those with expression in at most 5–10% of cells of all other types. This refined set of markers was then used to compute cell type scores for each cell type, per cell, using a MATLAB implementation equivalent to Seurat’s AddModuleScore. Pairwise scatterplots of the marker scores were inspected to determine thresholds above which cells were assigned to a cell type (Figure S3a). Cells with a single marker score above threshold were uniquely classified, while those that were above threshold in more than one cell type marker score were defined as ambiguous. A total of 1492 such ambiguous droplets were identified (4.6% of all droplets recovered), which is on the order of the expected number of doublets in the 10X system: ~6.4% per lane of 8000 cells, or a total of ~2000 cells total from four lanes (Zheng et al., 2017).

Ambiguous cells typically consisted of two contributing cell types. Analysis of the contributing cell types of ambiguous cells revealed that their numbers correlated with the total number of cells identified per cell type (Figure S2b, c). In UMAP embeddings showing marker scores and cell type classifications, several small distinct clusters of these cells could be identified between uniquely classified HPC clusters (Figure S4). Finally, a small number of cells could not be classified, having no cell type marker score above threshold (23 cells). To finalize the cell filtering, ambiguous cells and unclassified cells were excluded from further analysis, leaving 15,876 cells. An Upset plot indicating
the number of cells excluded for various quality control reasons is shown in Figure S5. The remaining unambiguously classified cell types corresponded very closely to UMAP clusters, confirming that cell-wise classification was successful.

2.5.5 | Ambient RNA removal with SoupX

We found significant contamination of droplets by ambient RNA, particularly for hormone genes such as Prl, Gh1, Pomc, and Cga (Figure S6). We quantified the contribution of genes to this ambient RNA with SoupX (Young & Behjati, 2020) and used SoupX-adjusted counts for differential expression analysis and visualization. Cell type identities were used as input clustering for SoupX and used the automatic estimation mode on each sample. The estimated global contamination fractions (rho values) were in 0.01–0.02 for all four samples, but we found that the contamination fraction needed to be set higher to remove hormone gene contamination; we used the upper value of rhoFWHM, which ranged from 0.039 to 0.047.

2.5.6 | Differential gene expression analysis

Differential gene expression analysis was done using a pairwise comparison approach on SoupX corrected counts to identify upregulated genes in cell types of interest relative to other cell types as previously described (Fletcher et al., 2019). We used the Kruskal–Wallis test (a nonparametric ANOVA-like extension of the Wilcoxon rank sum test to more than two groups) to identify whether a gene showed a difference in expression among cell types, followed by multiple comparisons to compute Bonferroni-adjusted p-values for each pairwise comparison (MATLAB’s kruskalwallis and multcompare functions). This pairwise analysis approach is closely related to the pairwise Wilcoxon rank sum test analysis available in scran (Lun et al., 2016). The Kruskal–Wallis and pairwise p-values were then corrected for multiple comparisons using the Benjamini–Hochberg method via the fdr_bh function (Groppe, 2021). To focus on genes with strong upregulation signals, we defined cell type-dominant genes as genes with expression in at least 20% of cells and with a significant upregulation (adjusted p-value < 1e-6) of at least 2.5-fold in normalized counts and a difference in proportion of expressing cells of at least +10%, relative to other cell types. Highly specific marker genes for a cell type were identified as above with the additional constraint that all other cell types had fewer than 5% of cells expressing the gene.

2.5.7 | Gene ontology and pathway over-representation analysis

Gene Ontology (GO) and pathway enrichment analysis was performed using the python implementation of gProfiler (Raudvere et al., 2019), with query gene sets for cell types of interest determined from differential gene expression analysis. We used unordered mode and the default p-value computation method (gscs) with a threshold of p = 0.05. For GO terms, we took advantage of the GO tree structure to remove redundant terms from the set of enriched terms. Redundant terms could be identified as larger, more generic terms with a set of genes enriched that was identical to one of its descendent terms, following only “is_a” relations (Jantzen et al., 2011). For visualization and to aid in interpretation of results from multiple queries, enriched terms were clustered using a gene-overlap approach. The sets of all unique terms and genes were identified across all queries, and a term-gene matrix was used to represent, for each term, the number of queries for which each gene was enriched. The Jaccard distance metric was then used for UMAP embedding of the terms (rows of the term-gene matrix), and subsequent clustering of the UMAP graph was performed using leidenalg. Rat orthologs for mouse gene symbols from marker lists were obtained using gProfiler’s orth tool (Raudvere et al., 2019).

2.5.8 | Ligand-receptor interactions using CellChatDB

To examine the possibility of ligand-receptor interactions between cell types, we used the approach of CellChat (Jin et al., 2021) with some modifications. We first converted the gene names in the CellChat database of ligand-receptor interactions from mouse to their rat orthologs using gProfiler’s orth tool. Next, we identified the set of genes from the database that were expressed in our dataset in at least 5% of cells of any cell type, which resulted in 459 genes representing 673 interactions. We normalized the expression of each gene to the maximum expression observed for that gene, then computed the mean normalized expression per cell type. The interaction strength for an interaction involving ligand L and receptor R was computed as \( \sqrt{L \times R} \), for every pairwise combination of cell types. If L or R were complexes, then the expression value for the complex was computed as the geometric mean of the expression of each member of the complex. We did not consider co-factors or activators and inhibitors, so as not to make any assumptions about the relationship between gene expression level, ligand and receptor protein levels, and ligand–receptor interaction affinities. Instead, we interpret the interaction strength as a measure of the potential for interaction, due solely to gene expression levels. Bootstrap p-values were computed as is done in CellChat. A null distribution of interaction strengths was generated from randomized expression profiles via shuffling of cell labels, repeated 10,000 times, and the observed interaction strength’s rank into this distribution was taken as the p-value.

3 | RESULTS

3.1 | The resident cell types of the pituitary gland

We performed scRNAseq (10X Genomics) on dispersed pituitary cells from 75-day-old random cycling female rats in three cell preparations.
from two pools of 30 animals: one from whole pituitary glands and the other two from separated anterior and intermediate/posterior pituitary lobes, respectively. After removing droplets with low genetic diversity and/or high fraction of mitochondrial counts (Methods, Figure S2), we classified individual cells using cell type-specific marker genes. This cell-wise classification approach allowed for identification of ambiguous droplets co-expressing marker gene profiles of more than one cell type, which could reflect doublets or multihormonal cells. Analysis of these droplets (4.6% of recovered droplets) suggested that they were likely doublets and not multihormonal cells, so they were removed from further analysis (Methods, Figures S3 and S4). After cell filtering based on quality control metrics and ambiguous droplets, we classified individual cells using cell type-specific marker genes with upregulation in FSCs and pituicytes relative to all other cell types. Nearly 1/3 of FSC-dominant genes were also expressed in pituicytes (Figure 2b, top panel). We then repeated the analysis using the same criteria, but comparing FSCs, pituicytes, pericytes, and ECs to each HPC and vascular cell type. To keep focus on FSCs and pituicytes, we show in Figure 2b (bottom panel) the Venn diagram of dominant gene counts with pericyte- and EC-dominant genes pooled together. We provide the full analysis for both comparisons in Supplemental Data 2 and 3. See also Figure S8 and Supplemental Data 4 for comparisons of anterior and posterior lobe-specific clusters of vascular cells, indicated in Figure 2b with the subscripts “a” and “p,” respectively.

Figure 2c highlights some of the highly cell-type-specific genes identified, and Figure 2d shows the expression level in individual cells on the UMAP plots for selected genes. Of the 301 uniquely pituicyte-dominant genes, over 1/3 were highly specific; they were expressed in less than 5% of cells in every other cell type (Figure 2c, top group). These genes were highly consistent with the previous report of pituicyte transcriptomes (Chen et al., 2020), accounting for 37 of the 80 genes listed in the latter including marker genes such as collagen type IX alpha chain 3 (Col9a3). Similarly, we identified several genes highly specific to FSCs (Figure 2c, second group). A quarter of FSC-dominant genes were highly specific, including the transcription factor regulatory factor X4 (Rfx4); Efnb3, coding for the endogenous ligand ephrin-B3; and aldehyde dehydrogenase 7 family member A1 (Aldh1a7). Expression of solute carrier family 6 member 11 (Slc16a11), coding for the sodium-dependent GABA transporter (GAT3), was also FSC-dominant. FSCs and pituicytes also co-expressed 46 dominant genes (Figure 2c, third group). These included the endogenous opioid polypeptide hormone proenkephalin (Penk), and family with sequence similarity 181 member B (Fam181b), which has recently been reported to interact with TEAD transcription factors, the downstream target of the Hippo signaling pathway (Bokhovchuk et al., 2020).
To illustrate the co-expression patterns among FSCs, pituicytes, and vascular cells, Figure 2c (bottom group) shows selected genes with high specificity to these cell types. FSCs, pituicytes, and pericytes of both pituitary lobes specifically expressed Hsd1b11, coding for hydroxysteroid 11-beta dehydrogenase 1; FSCs and pericytes expressed interferon induced transmembrane protein 1 (Ifitm1); and FSCs, pituicytes, and ECs expressed Gja1, coding for gap junction protein alpha 1 (connexin 43). These results, taken together with the above analysis, are suggestive of the numerous roles that have been ascribed to FSCs, including roles in metabolism and homeostasis, immune functions, gap-junctional coupling, and modulation of endocrine functions of the gland via endogenous ligands, as has been recently reviewed (Le Tissier & Mollard, 2021). Interestingly, several key genes that code for proteins implicated in these functions were coexpressed by vascular cells, particularly pericytes, suggesting the possibility that these cells may also contribute to the control of these functions. For example, Hsd1b11 and Anxa1, coding for annexin A1 (lipocortin I), have been implicated in FSC-mediated feedback of cortisol on the pituitary, and both shared expression among FSCs and pericytes. Finally, we note that many of the FSC-expressed genes highlighted in Figure 2 have been reported in astrocytes, as will be shown below.

### 3.2 | Astroglial signature of FSCs and pituicytes

#### 3.2.1 | Common and cell-type-specific expression of astrocyte genes

While pituicytes are considered astrocytes of the posterior lobe, there is no consensus regarding the classification of anterior lobe FSCs. Given the substantial shared expression of genes between FSCs and pituicytes, the classical literature regarding their morphology and functions, and the fact that S100b, Aldoc, and Slc15a2 (Figure 1) have all been reported as brain astrocyte markers, we examined the expression of astrocyte marker genes in these cells. To obtain a list of...
markers, we compiled published astrocyte-upregulated genes from seven references. These comprised three transcriptomic studies of cortical astrocytes from S100B-GFP transgenic mice (Cahoy et al., 2008), GFAP-GFP/GLT-1 positive astrocytes (Lovatt et al., 2007), and TRAP affinity-purified mRNA from BAC-transgenic Aldhl1l mice (Doyle et al., 2008); three scRNAseq studies, including astrocytes of the subventricular zone (Zywitza et al., 2018), and the cortex and hippocampus (Batiuk et al., 2020; Zeisel et al., 2015); and the astrocyte marker gene list from PangolaDB (Franzen et al., 2019).

For the former three, we used the normalized whole transcriptome data as provided by Ditte Lovatt and Nedergaard (2012), selecting all genes with log-fold-change >1.75 and adjusted $p < 0.01$, while for the single-cell studies we used the full list of reported astrocyte-upregulated genes. A set of 248 genes appeared in at least two of the seven references. Among these were 40 of 155 FSC-dominant genes and 64 of 347 pituicyte-dominant genes (identified in Section 3.1), 26 of which were expressed in both cell types (Supplemental Data 5).

Notably, Aldoc was reported in all seven references, along with claudin 10 (Cldn10), both dominant genes in FSCs and pituicytes.

To demonstrate the specificity of the astroglial signature of pituicytes compared to FSCs, we identified a focused set of 36 astrocyte marker genes appearing in at least four of the seven reference lists. Highlighted in Figure 3a are three FSC and pituicyte co-expressed genes: excitatory amino acid transporter 1 (EAAT1) gene, Slc1a3, or orphan G-protein coupled receptor Gpr37l1, the receptor for the secreted neuroprotective and glioprotective factor prosaposin (Meyer et al., 2013), and family with sequence similarity 107 member A (Fam107a); the pituicyte-specific gene Acyl-CoA synthetase, bubble-gum family, member 1 (Acsbg1); and the FSC-specific genes coding for phospholipase A2 group VII, Pla2g7, and aldehyde dehydrogenase 1 family member A1, Aldh1a1, coexpressed in some pericytes. The dot plot in Figure 3b shows the expression profile for 28 of these 36 astrocyte markers, which contained S100b, Aldoc, and Slc15a2. Additional notable astrocyte markers represented included Aqp4, expressed at
low levels in both cell types, pituicyte-specific Gfap, FSC-dominant NDRG family member 2 (Ndrg2), and Sox2 and Sox9, expressed well in both FSCs and pituicytes. The specificity of these astrocyte markers to FSCs and pituicytes is summarized in Figure 3c, showing the astrocyte score for every cell computed from the focused marker gene list.

We validated scRNAseq expression of astrocyte marker genes by performing qRT-PCR for 19 such genes in the whole pituitary, anterior lobe, and posterior lobe (Figure 3d). The qRT-PCR expression levels correlated well with the mean expression in the corresponding scRNAseq samples, providing support for our scRNAseq findings.

Genes tested included S100b, Sox2, Gfap, Aldh1a1, Aldh111, Aap4, Edn3, Fox1, Fgfr3, Fibin, Gstm7, Mael, Nes, Penk, Slc1a2, Slc1a3, Sox9, Sult1a1, and Vim.

3.2.2 | Expression of S100 family members in pituitary cells

S100B, a protein marker of astrocytes (Brozzi et al., 2009), has historically been viewed as a definitive marker for FSCs in the rat pituitary
We show here that S100b is a good marker gene for both FSCs and pituicytes; only a small number of vascular cells and melanotrophs show low expression of this gene (Figure 4a and b). In total, we observed S100b expression in 2388 of 14,309 analyzed anterior pituitary cells, representing 16.7% of cells in this lobe. S100b is a member of the S100 family of genes (Donato...
et al., 2013), but the expression and role of other members of this gene family have not been characterized in pituitary cells. Here, we show that pituitary cells also expressed S100a1, S100a6, S100a10, S100a11, S100a13, S100a16, and S100g genes (Figure 4a and b). S100a1 had a similar expression pattern but was better expressed in FSCs than pituicytes. S100a6 and S100a11 were highly specific to FSCs and pericytes, but not pituicytes, with stronger expression in anterior lobe pericytes. S100a10 was similarly well expressed in FSCs and pericytes, but also exhibited lower-level expression among ECs, and S100a10 and S100a11 had some low-level expression in HPCs. S100a13 was broadly expressed, but with highest levels in FSCs and pericytes, while S100a16 was localized to all non-HPCs, predominantly ECs and FSCs. Finally, S100g had a unique pattern of expression, specifically localized to pituicytes of the posterior lobe and lactotrophs in the anterior lobe, with low levels detected in some somatotrophs and gonadotrophs (Figure 4a and b).

To confirm the expression of these S100 family members in the pituitary, we performed independent qRT-PCR and immunofluorescence analysis. qRT-PCR showed good agreement between dispersed and intact tissue samples for S100B and the six S100A family members described above (R² = 0.839), indicating that cell dispersion did not disrupt expression of these genes (Figure 4c). Immunohistochemistry analysis of pituitary tissue of female rats revealed the expression of S100B in both the anterior and posterior lobes (Figure 4d and e). In the anterior lobe, out of 13,670 analyzed cells, 1860 cells were S100B positive, representing 13.6% of cells, which is comparable with the percentage of S100b-positive cells observed in scRNAseq study. S100A-positive cells were also observed, and the overlay clearly indicates the coexpression of S100A and S100B in cells from the anterior (Figure 4d) and posterior (Figure 4e) pituitary lobes. Note that the S100A antibody does not separate between subtypes of A subfamily of these proteins. In the primary cultured pituitary cells from both male and female animals, S100A and S100B immunofluorescence was also localized to cells with prominent projections, characteristic of FSCs (Figure S9). Colocalization of S100A and S100B occurred in 56% of S100A+ cells and 81% of S100B+ cells, counted from a total of six separate replicates, consistent with the scRNAseq result that cell populations other than FSCs, including pericytes, expressed S100A gene family members but not S100b.

3.2.3 Expression of other astroglial proteins in FSCs and pituicytes

S100B protein is expressed in astrocytes but it has also been reported in other cell types (Donato et al., 2013). In further immunohistofluorescence studies, we used antibodies against reliable astrocyte markers: EAAT1/SLC1A3, ALDOC, ALDH1A1, and VIM. EAAT1 is an astrocyte glutamate transporter that contributes to the uptake of glutamate in the brain. It belongs to the carrier 1A family of transporters (Todd & Hardingham, 2020). SLC1A3 was well expressed in FSCs and pituicytes and was also detected at low levels in the lactotroph fraction (Figure 3b). Immunopositive EAAT1/SLC1A3 cells were detected in both anterior and posterior pituitary sections (Figure 5). In anterior pituitary sections, most EAAT1-positive cells also expressed S100B, but there were a few S100B-positive cells that did not express EAAT1, probably some vascular cells that expressed S100B (Figure 5, top panels). No EAAT1-positive and S100B-negative cells were detected, further indicating that the expression of this transporter in lactotrophs was probably below the detection by immunohistofluorescence. In the posterior pituitary, both S100B and EAAT1 were strongly expressed, and most pituicytes co-expressed both proteins (Figure 5, bottom panels).

Alaldase, fructose-bisphosphate C (ALDOC) is also an established astrocyte marker protein (Levine et al., 2016). Its gene Aldoc is well expressed in FSCs and is also present in pituicytes but at lower expression levels (Figures 1d and 3b). In anterior pituitary sections, virtually all S100B-positive cells co-expressed ALDOC (Figure 6, top panels), consistent with prior reports (Fujiwara et al., 2020). Aldehyde dehydrogenase 1A1 (ALDH1A1) is another protein specifically expressed in astrocytes (Kwak et al., 2020) and we observed Aldh1a1 expression specifically in FSCs and pericytes (Figure 3a, b). Coexpression experiments with S100B and ALDH1A1 confirmed the presence of this protein in FSCs; most cells expressed both proteins (Figure 6, bottom panels). We also examined the expression of vimentin (VIM) in the anterior and posterior pituitary sections. VIM is a specific intermediate filament protein expressed in astrocytes and ependymal cells of neuroectoderm (Schnitzer et al., 1981). In the pituitary gland, VIM is expressed in FSCs, pericytes, endothelial cells, and at lower level in pituicytes (Figure 3d). VIM-immunopositive cells were also detected in both anterior and posterior pituitary sections (Figure S10; arrows show cells co-expressing both proteins). Taken together with the scRNAseq expression data, these results indicate that FSCs and pituicytes are astroglial cell types that express a number of common and some cell-type-specific astrocyte markers.

3.3 Heterogeneity of FSCs

3.3.1 Two subclusters of FSCs

We previously observed two subclusters of FSCs in both male and female samples (Fletcher et al., 2019), so we investigated the heterogeneity of FSCs here by unsupervised clustering of these cells. Two subpopulations were identified, one larger (FS1, 1877 cells) and one smaller (FS2, 511 cells) (Figure 7a). Interestingly, FS2 cells expressed a much greater diversity of genes compared to FS1, with a median of 3137 and 2099 genes detected per cell, respectively (Figure 7b). This suggests that FS2 cells possess increased differentiation potential (Gulati et al., 2020). The FS2 cluster also contained a small subcluster of cells expressing cell cycle marker genes (Figure 7a, circled), accounting for the same proliferative FSCs circled in Figure 1a.

To find shared and distinct FSC marker genes, we again used pairwise differential expression analysis (Methods) to identify FS1- and FS2-upregulated genes relative to all HPCs, vascular cell types, and leukocytes; we exclude pituicytes to allow comparison with these cells.
in the following section. This analysis identified 79 genes dominantly coexpressed by both FS1 and FS2 (pan-FSC), 21 FS1-dominant genes, and 201 FS2-dominant genes (Figure 7c, Supplemental Data 6). The fact that ~80% of FS1-upregulated genes are also upregulated in FS2 suggests that FS1 and FS2 cells are closely related cells, consistent with their close co-clustering in the UMAP embedding relative to other cells (Figure 1b). In contrast, only ~30% of FS2-upregulated genes were pan-FSC genes, suggesting that these cells have additional activated gene expression programs. Figure 7d highlights some highly specific FSC-expressed genes, and the UMAP plots in Figure 7e shows the expression level of selected genes in individual cells. Notable pan-FSC genes included tissue inhibitor of metalloproteinase 1 (Timp1), consistent with the previous reports (Azuma et al., 2015), and flavin-containing monoxygenase 1, Fmo1. FS1-dominant genes included guanylate cyclase 1 soluble subunit alpha 1 (Gucy1a1), angiotensin (Agt), and C-X-C motif chemokine ligand 1 (Cxcl1). The latter two genes indicated localization to restricted parts of the UMAP cluster of FS1 cells, suggesting possible specializations of these cells. FS2-dominant genes included Tent5b, which encodes terminal nucleotidyltransferase 5B, Krtn17, encoding the intermediate filament keratin 17, and the P2Y purinoreceptor 2, P2ry2. This analysis offers a list of candidate genes of interest for FSC subtypes, listed for reference in Supplemental Data 6. The "stem-like FSC gene score" panel in the table of contents image summarizes the set of FS2-upregulated genes that were also dominantly expressed relative to FS1.

We also noticed that FSCs shared expression of several genes with HPCs. However, a majority of these were dominantly expressed by FS2 compared to FS1 (Figure 7d, bottom group). Differential expression analysis identified 65 genes upregulated in FS2 and at least one HPC cell type relative to all other non-HPC types (including FS1), while only one such gene was identified for FS1. Some examples of these genes included: Krt8 and Krt18, cytokeratins which have been associated with PSC niches (Shintani & Higuchi, 2021); Rab25, which encodes low molecular weight GTPase with preferential expression in the pituitary gland (St-Amand et al., 2011) and has been linked to cell migration in tumor metastasis (Caswell et al., 2007); a potassium two pore domain channel gene Kcnk1; Epcam, which encodes the epithelial cell adhesion molecule, reported to be expressed in nonfunctional pituitary adenomas (Wang et al., 2019); and tight-junction associated claudins, Cldn9 and Cldn3. A total of 42 genes expressed in both HPCs and FSCs did not exhibit FSC subtype-dominant expression, including expected genes such as Pitx1 and Pitx2, again suggestive of their close relationship.
To determine if the two FSC subtypes differentially expressed astrocyte marker genes, we compared the expression of these genes among FS1, FS2, and pituicytes (Figure S11). While a small number of genes showed deviations, the expression levels of astrocyte genes and the proportion of expressing cells among FS1 and FS2 were highly correlated (expression $R^2 = 0.78$, proportion $R^2 = 0.75$; top panels, Figure S11). Conversely, there was very low correlation between FS1 or FS2 and pituicytes ($R^2 < 0.1$; middle and bottom panels, Figure S11). These observations support the close relationship between the FSC subtypes, and that while both FSCs and pituicytes express astroglial genes, they have lobe-specific specializations.

### 3.3.2 FS1 and FS2 cells: Common and distinct signaling pathways

To gain insight into the roles suggested by FS1- and FS2-genes, and to compare them with pituicyte-dominant genes (Section 3.1), we tested each of the three sets of genes for enrichment among Gene Ontology terms (BP, biological process; CC, cellular component; and MF, molecular function) and pathways (KEGG, Kyoto Encyclopedia of Genes and Genomes; REAC, Reactome) using gProfiler (Raudvere et al., 2019) (Methods). The results for the three tests were then compiled, resulting in a total of 315 significant terms (adjusted $p < 0.05$) for at least one of the three cell groups. The full set of results, including the lists of enriched genes per term, are provided in Supplemental Data 7. For visualization of significantly enriched terms, we used UMAP embedding and clustering, using the overlap among the sets of enriched genes for each term as a metric for term similarity (Methods). The resulting UMAP embedding of terms, colored by cell type combinations, is shown in Figure 8a with the colors of the intersections in the Euler diagram serving as the color legend. Clustering was used to group together terms with the similar sets of enriched genes (Figure 8b), and the enrichment test results for representative terms from each cluster are shown in Figure 8c.

Clustering revealed groupings of terms suggestive of overlapping and distinct functions for pituicytes and FSCs, including metabolism and detoxification, developmental processes, and morphogenesis, signaling, extracellular matrix, and cell junctions. Clusters 1 and 2 pointed to the functions of pituicytes and FSCs relating to metabolic processes and detoxification, as well as multiple types of metabolite and...
ion transporters in pituicytes. Many enriched terms related to developmental processes and control of cell proliferation (clusters 3–7). Cluster 3 suggested roles for FS1 in promoting differentiation of cells, while FS2 genes were enriched instead in terms related to signaling pathways involved in regulation of stem cell functions, such as Hippo and Wnt. Cluster 4 captured terms suggestive of FS2 cell participation in tight junctions/apical junction complexes, as well as anchoring junctions for both pituicytes and FS2 cells. This observation, along with FS2 enrichment for terms related to epithelium development, is suggestive of FS2 cell involvement in the structural aspects of the parenchymal or marginal cell layer stem cell niches. Cluster 5 contained FS2-enriched terms related to cell migration, development, and positive regulation of cell proliferation, further suggestive of stem cell/niche activity. Cluster 6 featured terms enriched for both FS1 and FS2 genes, pointing to shared involvement in the regulation of differentiation of astroglial cells and HPCs, gland morphogenesis, and general regulation of cell proliferation. Finally, cluster 7 pointed to pituicyte-specific roles involving collagen-containing extracellular matrix, developmental processes unique to the posterior lobe, and more generic shared terms relating to CNS development, axon guidance, and cell adhesion.

The enrichment of FS2 genes in Hippo and Wnt signaling pathway terms (cluster 3, Figure 8) is consistent with observations that these pathways are important in PSC functions (Lodge et al., 2016; Russell et al., 2021). Motivated by these results, we examined expression of all genes from the KEGG Hippo signaling pathway term (KEGG:rno04390), focusing on their expression in anterior lobe cells. Ninety pathway genes had expression in at least 15% of either FS1 or FS2 (Supplemental Data 8). However, the proportion of cells per subtype expressing these genes was at least 10% greater in FS2 than in FS1 for 81 of these, suggesting a higher degree of Hippo pathway related activity in FS2. We highlight the expression profiles of key pathway genes for anterior lobe cells in Figure 9a, where $P_{E_{0}}$ and $E_{C_{0}}$ represent the anterior lobe clusters of pericytes and endothelial cells. Both Yap1 and Taz (Wwtr1), the main coactivators of the pathway, were better expressed in FS2 compared to FS1. FS2 also expressed genes coding for the transcription enhancer factor (TEAD/TEF) family of transcriptional factors ($\text{Tead2}$ and $\text{Tcf7l1}$). Several other downstream Yap1 activated target genes were upregulated in FS2 compared to FS1, including $\text{Ccn1}$, $\text{Myc}$, $\text{Ccn2}$, $\text{Id1/2}$, and $\text{Nkd1}$. FS2

FIGURE 7 Identification of two FSC subtypes. (a) UMAP embedding showing unsupervised clustering of FSCs into two subtypes. A small portion of FS2 cells (circled) express cell cycle markers. (b) Violin plot of the distributions of number of genes per cell in FS1 and FS2, with median values of 2099 and 3137, respectively. (c) Venn diagram showing the number of genes identified as FS1-dominant, FS2-dominant, and common to both, relative to other cell types except pituicytes (full results in Supplemental Data 4). (d) Dot plot showing selected top FSC-expressed genes: dominant in both subtypes (first group), FS1-dominant (second group), FS2-dominant (third group), and co-expressed in FS2 and HPC (fourth group). (e) UMAP scatter plots showing the expression patterns of selected genes from panel d.
also expressed genes suggesting regulation of Hippo signaling by cell density via tight junctions or adherens junctions in these cells, including cell junction associated genes *Pard6b*, *Amot*, *Amotl2*, and *E-cadherin (Cdh1)*, the latter also noted as a PSC marker gene (Batchuluun et al., 2017; Fauquier et al., 2008; Yoshida, Nishimura, et al., 2016). This is consistent with our gene expression and enrichment analysis indicating that these cells likely participate in cell junctions, characteristic of dense parenchymal FSC clusters or the marginal cell layer. Several Hippo-regulating signaling pathway genes were also well expressed. *Wnt5a* ligands were more highly expressed by FS1, while FS2 expressed Fzd receptors at higher levels, most notably *Fzd3* and *Fzd1*, suggesting FS1 to FS2 signaling. BMP signaling pathway elements were more highly expressed in FS2, including *Bmp7*, *Bmpr1a*, and *Bmpr2*, suggesting possible autocrine BMP signaling.

Based on the previous reports of involvement of Wnt, BMP, FGF, and Notch signaling in PSC functions (Batchuluun et al., 2017; Chen et al., 2006; Lodge et al., 2016; Nantie et al., 2014; Russell et al., 2021; Zhu et al., 2015), we tested the possibility of FSC-targeted signaling by these pathways, as well as cell–cell interactions involving *Cdh1*, using an approach for estimating ligand-receptor interactions based on CellChat (Methods) (Jin et al., 2021). We focused here on FSC-targeted interactions involving vascular cells or FSCs as the source, but interactions with HPCs were also detected (See Supplemental Data 9). Selected results are shown in the dot plot in Figure 9b, which indicates potential strength of interaction (color) as well as the percentage of cells of the target cell type that expressed the receptor (size). We note that this analysis shows only the possibility for interaction based on ligand and receptor gene expression levels; a functional interaction requires functional ligand and receptor proteins and a permissive spatial and temporal association between source and target cells. Among the strongest interactions identified was Notch 2 signaling in FS2 cells, driven by EC-expressed Dll4 ligands or pericyte-expressed Jag1 ligands. Strong potential interactions involving homotypic Ncam1 interactions among FSCs were identified, with FS1 having double the expression level of Ncam1 compared to FS2. Ncam1-Fgfr1 interactions directed from FS1 to FS2 are also suggested due to higher FS2 expression of Fgfr1. We note that these genes were also expressed in some HPCs; Ncam1 primarily in corticotrophs, thyrotrophs with lower levels in other HPCs, and Fgfr1 in Pou1f1-expressing cells. The strongest interaction among potential Hippo regulating pathways involved FS1 signaling to FS2 via Wnt5a
and Fzd1 or Fzd3 receptors, as well as homotypic FS2 Cdh1 cell–cell interactions. Besides Wnt5 signaling, further Wnt signaling interactions may be mediated by EC-expressed Wnt2, as well as a possible Wnt9a autocrine interaction between FS2 cells. The possibility of BMP signaling was strongest via EC-expressed Bmp4 and pericyte-expressed Bmp2 directed towards FS2, followed by the possibility of Bmp7 autocrine signaling in FS2 cells. The analysis suggests BMP ligands may act via FS2-expressed Bmpr1a+Bmpr2 receptor complexes, or possibly Bmpr1a+Acrv2a or Acrv2b, the latter two genes being expressed at lower levels than Bmpr2. Finally, we also identified a possible autocrine signaling of FS2-expressed Fgf9 or Fgf1 via Fgfr1 or Fgfr2, as well as a weaker potential interaction with FS1-expressed Fgfr3.

Taken together, these results suggest that FS2 cells are likely to be members of stem cell niches, and that this cluster may contain active stem cells and/or HPC-committed progenitors, while FS1 cells represent a differentiated FSC cluster.

3.3.3 Stem/progenitor and differentiation marker gene expression

We next investigated the expression of stem/progenitor and differentiation marker genes in the anterior and posterior pituitary cells. UMAP embeddings for six key genes are shown in Figure 9c. Sox2 is generally accepted as a marker gene of the anterior pituitary stem
cells (Kelberman et al., 2006). However, of the 14,309 anterior pituitary cells analyzed, 2480 cells were Sox2-positive, representing ~16.8% of cells in this lobe, a percentage unlikely for stem cells. This gene was expressed in FS2, as expected, but also in FS1 and pituicytes, as well as in a fraction of HPCs at low levels (Figure 9c and d). These results clearly show that Sox2 expression is not sufficient the specific identification of stem cells. Sox9 is another proposed stem cell gene (Rizzoti, 2010), which is also expressed in FS1 and FS2, as well as in pituicytes. The same expression pattern is shown for Ntrk2 and Six3. Other known stem cell marker genes, including Vim, Cd9, and Hes1, were well expressed not only in FS1, FS2, and pituicytes, but also in vascular cells (Figure 9d).

The similarity in expression of developmental genes in FSCs and pituicytes raised the possibility of their common embryonic development. However, pituicytes also uniquely expressed many genes, including expected markers Fgf10, Tbx3, Lhx2, Rev, and Nkx2. These findings indicate developmental/differentiation processes specific to the posterior lobe, at least during the postnatal period. FSCs also specifically expressed several stem cell-like genes, including Prop1, Hey1, Hey2, and Msx1, and shared expression of Prx1 and Heyl with pericytes, further supporting the conclusion of separate postnatal proliferation of these two cell types. FSCs, but not pericytes, also expressed several differentiation marker genes with HPCs, such as Isl1, Cd24, Six1, Six6, Pitx1, Pitx2, and Lhx3. Finally, HPCs but not FSCs, express some differentiation genes in a cell-specific manner, including Tbx19, Nr5a1, Gata2, and Pou1f1. Such a pattern of expression of differentiation genes confirms the dependence of postnatal HPC proliferation on FSCs.

### 3.3.4 Immunohistofluorescence characterization of pituitary stem cells

The expression of SOX2 protein was observed in the anterior, intermediate, and posterior lobes of the pituitary gland, as well as in the marginal zone between the anterior and intermediate lobes (Figure 10). In the anterior and intermediate lobes, SOX2 was randomly expressed, predominantly in S100B-positive cells, indicating that FSCs expressed this protein. Consistent with the expression of Sox2 in the fraction of HPCs (Figure 9c and d), some cell types that did not express S100B were immunopositive for SOX2 (indicated by arrows in Figure 10). In the marginal zone, SOX2 positive cells formed a stream-like band. These cells were also S100B-positive, confirming their identity as FSCs. In the posterior lobe, the coexpression of SOX2 and S100B was consistent with finding that pituicytes also express S100B protein (Figure 4e). The contribution of SOX2-positive cells to anterior pituitary lobe was 14.7% (1748 of 11,921 cells), which again indicates that all FSCs are unlikely to be stem cells.

To clarify the spatial location of FS2 cells, we used double labeling between S100B and the gene products of two identified FS2-specific genes, KRT17 and TENT5B (Figure 7d and e). Unlike SOX2, which was present in a cell fraction in all three pituitary lobes as well as in the marginal zone, all KRT17-positive cells were located exclusively in the cell band of the marginal zone and these cells coexpressed S100B protein (Figure 11). Note that S100B positive cells also form a separation wall between intermediate and posterior lobes, but these cells were KRT17-negative, as well as S100B-positive cells in the anterior and intermediate lobes (Figure 11). KRT17-positive cells were also
SOX2-positive, but the marginal zone band also contained SOX2-positive and KRT17-negative cells (Figure S12). The Tent5b gene was exclusively expressed in FS2 cells (Figure 7d and e), and TENT5B-positive cells were also found in the marginal zone between the anterior and intermediate lobes (Figure S13).

Together, these data show that only the FS2 cell cluster contains stem cells, that differentiated FS1 cells also express SOX2, and that a fraction of HPCs, which are probably a recently developed cell population in which the SOX2 gene that has not yet been silenced.

4 | DISCUSSION

We used here scRNAseq, qRT-PCR, and immunohistofluorescence to investigate the transcriptomic profiles of anterior and posterior pituitary cells obtained from adult random cyclic female rats. We identified six HPC types: melanotrophs, corticotrophs, gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs; four nonhormonal cell types: FSCs, pituicytes, and vascular ECs and pericytes; and blood cells: leukocytes and erythrocytes. A small population of cells expressing proliferation marker genes was also identified. However, our cluster analysis did not identify a distinct small cluster of Sox2-positive adult PSCs. The anterior pituitary cell identification in this study was highly comparable with our previous work (Fletcher et al., 2019), but the percentage of specific pituitary cell population was somewhat different, probably reflecting the impact of cell dispersion procedures. In this study, combined and separate scRNAseq analysis of anterior and posterior lobe tissue served as an internal control to confirm the anterior lobe specificity of FSCs, the posterior lobe specificity of pituicytes, and the dominant separation of melanotrophs of the intermediate lobe with the posterior lobe. More importantly, it directed us to four major lines of investigations: the presence of lobe-specific clusters of vascular cells, the astroglial nature of FSCs and pituicytes, the co-expression of S100B and SOX2 in FSCs and pituicytes, and the transcriptomic and functional heterogeneity of FSCs.

Vascular cells are the least characterized group of pituitary cells, but their relevance in pituitary functions other than blood supply has recently been raised (Le Tissier et al., 2017). Their characterization at the single-cell level, including in scRNAseq studies, has received little attention, although methods for isolating rat pituitary endothelial cells (Chaturvedi & Sarkar, 2006) and scanning electron microscopy of pericytes (Jindatip et al., 2012) and EC (Itoh et al., 2003) have been established. Our scRNAseq study points to two major characteristics of vascular cells. Firstly, there is shared expression of some genes in pericytes and FSCs, including the expression of receptor ligand genes Mdk, Ptn, and Edn3. Secondly, we observed the lobe-specific clusters of vascular cells, reflecting a long list of genes differentially expressed in the anterior or posterior pituitary gland. These findings should open the field for further investigations, because only a few preliminary data are available at the present time, including the expression of Des (Jindatip et al., 2021), laminins (Ramadhani et al., 2012), and collagen (Fujiwara et al., 2010) in vascular cells.

The transcriptome profile of rat pituicytes described here is highly comparable to that reported in mouse pituicytes (Chen et al., 2020) but we provide additional information about their transcriptomes and relationship with FSCs. Pituicytes are a generally accepted subtype of astrocyte (Verkhratsky & Nedergaard, 2018), while FSCs have been recognized as astrocyte-like cells (Osuna et al., 2012). S100b is a good marker gene for rat FSCs and brain astrocytes (Osuna et al., 2012), as well as for pituicytes (Chen et al., 2020), motivating us to examine to expression of this gene and protein in pituitary cells. S100 proteins are a multigenetic family of low molecular weight calcium binding proteins, consisting of multiple S100A proteins, S100G, S100P, and S100Z, in addition to S100B (Donato et al., 2013). S100b-positive
pituitocytes were reported to appear in the posterior lobe on rat embryonic day 15.5 and in the anterior lobe on embryonic day 21.5 (Horiguchi et al., 2016). Several reports indicate S100G proteins in lactotrophs and GH3 immortalized lacto-somatotrophs (Nguyen et al., 2005; Vo et al., 2012). Here, we confirmed that S100G is expressed in lactotrophs, as well as in pituitocytes and a fraction of somatotrophs and gonadotrophs, but not in FSCs. The expression of S100A genes has not been previously assessed in the anterior and posterior pituitary gland. Our data indicate that FSCs and pituicytes express S100a1 and S100a16, the latter being also expressed in ECs and pericytes. Both genes have been reported as astrocyte-upregulated genes (Ditte Lovatt & Nedergaard, 2012; Zwytitz et al., 2018). S100a6, S100a10 and S100a11 are expressed in FSCs and pituicytes, but not in pituitocytes. These data provide a basis for further investigations into the role of these proteins in pituitary functions and indicate similarities and differences in the expression of these genes in FSCs and pituitocytes.

However, due to the expression of the S100B gene in other cell types (Donato et al., 2013), these data alone cannot be used to address the astrocytic nature of FSCs. In further work on this topic, we characterized gene expression profiles in FSCs and pituitocytes, focusing on astrocyte marker genes. We identified astrocyte-upregulated genes from six transcriptomic studies and the PangolDB astrocyte marker gene list, focusing on genes appearing in at least two of the seven references. These astrocyte genes were highly represented among both FSC- and pituicyte-dominant genes, supporting the classification of these cell types as pituitary astroglial cells. As mentioned above, both cell types expressed several genes present in brain astrocytes: Mdk, Ptn, Penk, Sox2, Sox9, Cd9, Vim, Hes1, Six3, Ntrk2, Gpr37l1, and S100b, as well as Aldoc, Apoe, Aqp4, Gja1, Glul, Maob, Phgdh, and Slc1a3, additional astrocyte marker genes (Verkhratsky & Nedergaard, 2018). FSCs and pituitocytes also expressed additional astrocyte markers present in brain astrocytes: C1r, Cym, Gstm1, Cybrd1, Gas, Gpm6b, Id4, Lcn2, Metn, Mxra8, Nqo1, Pchd7, Plat, Slc20a2, Slit2, Sod3, Tthy1, Tfgb2, Wnt5a, and Fzd1. FSCs, but not pituitocytes, expressed: Aldh1a1, Aldh1a2, Aldh3a1, AnxA1, Cryab, Cxxc1, Fibin, Hey2, Hspb8, I133, Mx1, and Slc6a8. Finally, we observed low levels of Glap expression uniquely in pituitocytes. The gene scores based on these genes uniformly labeled both FSC and pituicyte clusters, supporting the common identity of these cells as pituitary astrocytes.

We also used an immunohistochemical approach to study the expression of known astrocyte marker genes in pituitary cells: ALDOC (Levine et al., 2016), ALDH1A1 (Kwak et al., 2020), EAAT1/SLC1A3 (Todd & Hardingham, 2020), and Vim (Schnitzer et al., 1981). Most S100B-positive cells in the anterior and posterior lobes coexpressed ALDOC, ALDH1A1, and EAAT1; a few S100B-positive cells that did not express these proteins were probably vascular cells that also expressed S100B. Consistently with these findings, Aldoc has recently been proposed as a good marker for FSCs in both mouse and rat (Fujigawa et al., 2020). Immunopositive VIM-positive cells were also detected in the anterior and posterior pituitary cells. A lower percentage of VIM-positive cells may be related to the level of expression of this protein and the sensitivity of the antibody used to detect it. Taken together, these three lines of evidence clearly show that FSCs and pituitocytes belong to astrocytes, expressing common and cell-type-specific astrocyte genes.

Among the astrocyte marker genes, Sox2 has a somewhat unique role. It is expressed in the adult neurogenic niche (Suh et al., 2007), but is also detectable in proliferating astrocytes in the developing mouse brain, as well as in the early postnatal astrocytes in culture (Bani-Yaghoub et al., 2006). In postnatal pituitary gland, it is generally accepted as a stem cell marker gene (Zhu et al., 2015). However, the recent scRNAseq studies of pituitary glands have reported inconsistent findings regarding stem cell versus FSC identity. The first scRNAseq study of whole mouse pituitary cells identified a Sox2-positive cluster as stem cells but did not identify FSCs in their cell type classification (Cheung et al., 2018). The transcriptome profile reported for the Sox2-positive cluster did not list other known stem cells genes, such as Cdh1, Cdx9, Lhx3, Prop1, Prx1, Vim, Sox9, and S100b (Garcia-Lavandeira et al., 2012; Gielberman et al., 2008; Horiguchi et al., 2018; Rizzoti, 2010; Vankelecom & Chen, 2014), but instead included Aldh1a2, Aldh3a1, Aqp4, Cdhd2, Cxpm2, Lcn2, and Rbpms (Cheung et al., 2018). Here and in our prior scRNAseq study (Fletcher et al., 2019), these genes were expressed in FSCs, and of these only Cxpm2 was specific to FSC2.

Another mouse pituitary scRNAseq study also labeled Sox2-positive cells as stem cells and did not identify FSCs (Lopez et al., 2021). A third study did separately label Sox2-positive cells from FSCs (Ho et al., 2020). However, their Sox2-positive cells co-express Aldoc, Crlf1, Phgdh, Nrcam, and Mgst1, common FSC genes, while their FSC cluster expressed Pdgfrb, Dcn, Lum, Col1a1, and Col3a1, which are established marker genes of pericytes. No pericyte cluster was identified in their study, which could explain this difference. The recent scRNAseq study with mouse pituitaries identified two stem cell clusters but did not classify any cells as FSCs (Vennekens et al., 2021). However, it is known that S100b is a poor marker gene in the mouse pituitary, which could contribute to problems with identification of mouse FSCs. Finally, cell-type differences in survival through cell dispersion may contribute to the wide variation in proportion of Sox2-positive cells/FSCs among scRNAseq studies, which have ranged from ~2.5% (Cheung et al., 2018) to ~30% (Fletcher et al., 2019).

The expression of Sox2 in FSCs is consistent with a long-standing hypothesis that these cells have stem cell capabilities (Inoue et al., 2002). However, renaming FSCs as Sox2-positive stem cells seems unreasonable. Firstly, FSCs are an established anterior pituitary cell lineage with numerous specific functions reported during a long history of studies (Le Tissier & Mollard, 2021). These include their capacity to act as phagocytes, immune cells and cells that regulate hormone release (Allaerts & Vankelecom, 2005; Inoue et al., 1999), as well as for generation of numerous endogenous ligands, detoxification enzymes, extracellular matrix proteins, and cell adhesion molecules (Fletcher et al., 2019). These functions are critical for the proper tridimensional organization and function of the anterior pituitary gland, which includes the FSC gap-junction coupled network (Fauquier et al., 2001).
Secondly, we showed here that Sox2, the putative stem cell marker gene of the anterior pituitary, is expressed not only by FSCs, but also by pituicytes and at lower levels by some HPCs, including melanotrophs and corticotrophs, consistent with our previous experiments done with anterior pituitary cells from adult male and female rat (Fletcher et al., 2019) and the previous immunohistofluorescence studies (Kato et al., 2021). Practically, 17% of the anterior pituitary cells were Sox2-positive, and 14.7% were SOX2-immunopositive. Our immunohistofluorescence analysis of pituitary tissue also confirmed the expression of SOX2 in both S100B-immunopositive and negative cells in the anterior and intermediate lobes, as well as in the posterior pituitary. SOX2-immunopositive cells were also detected in a well-organized band of cells in the marginal zone between the anterior and the intermediate lobes, previously identified as the stem cell-containing region (Castinetti et al., 2011). Therefore, Sox2 cannot be accepted as the sole marker gene for PSCs.

These contradictions led us to investigate the heterogeneity of FSC. Our analysis suggested that these cells form at least two subclusters. FS1 contained more cells (~80%) but less genetic diversity, as opposed to FS2 with fewer cells (~20%) but with a 50% higher median number of genes expressed per cell, suggesting that they have higher differentiation potential (Gulati et al., 2020). Such expression profiles suggest a classical FSC role for FS1 cells, including expression of enzymes, transporters, and shared pan-FSC marker genes. In contrast, the transcriptomic profile of FS2 included additional genes suggestive of an epithelial-like polarized cell phenotype expected for cells participating in dense parenchymal FSC clusters and/or marginal cell layer associated with stem cell niches. Our further analysis revealed that FS1 and FS2 clusters do not contain subclusters (not shown), despite their multiple reported roles in the pituitary gland. Instead, we observed smooth changes in expression across this subcluster (e.g., Cxcl1 and Agt, Figure 7e), suggesting that FS1 cells may be equipped to perform multiple roles based on the degree of expressed genes.

Several additional findings indicate that FS2 may represent active stem/progenitor cells in anterior pituitary. Proliferative FSCs were clustered with FS2 and proliferative FSCs were observed in our previous scRNAseq data (Fletcher et al., 2019), suggesting a role for these cells in the cell proliferation required to generate HPC-committed cells and/or renewal of stem cells. Several FS2-specific Gene Ontology terms were related to development, including positive regulation of cell proliferation, and stem cell pluripotency regulation. FS2 specifically expressed genes coding for tight junction proteins and E-cadherin, consistent with the idea that these cells were members of the parenchymal and/or marginal zone stem cell niches (Higashi et al., 2021). This analysis also identified FS2-specific terms for cell migration, possibly reflecting the epithelial-mesenchymal transition needed for release of new cells from stem cell niches (Yoshida, Kato, et al., 2016). Our analysis revealed that FS2 shared expression of genes with HPCs, suggestive of cells in transition to HPCs. Hippo signaling has been shown to be important in PSC maintenance (Lodge et al., 2016), and a large complement of these genes were more highly expressed in FS2. Finally, our CellChat-based ligand–receptor interaction analysis identified preferential signaling towards FS2 cells via several signaling pathways known to be important in PSC function, including Notch, Wnt, BMP, and Fgf.

The presence of FS1 and FS2 cells, both expressing Sox2, raised the question of their localization in the anterior pituitary. To address this, we looked for genes expressed only in FS2 cells and available antibodies against the proteins generate by these genes; two such genes/proteins were identified: KRT17 and TENT5B. Immunopositive KRT17 and TENT5B cells were identified within S100B and SOX2-positive cells, but only in a fraction of these cells located in the marginal zone, the starting point proposed for the location of adult stem cells in the pituitary gland (Castinetti et al., 2011). The presence of SOX2-expressing cells near cells expressing FS2-specific markers in the marginal zone, which could be newborn cells, and the presence of SOX2 and S100B genes and proteins in a fraction of HPCs further supports the idea that FS2 cells serve as stem cells for HPC regeneration. We suggest that newborn cells differentiate into HPCs or FS1, but with distinct differentiation processes. During HPC differentiation, most developmental/differentiation marker genes cease to be expressed, while during FS1 differentiation these genes continue to be expressed, implying that these genes may be required for FS1 cell function. Another possibility is that SOX2 expression in differentiated FS1 cells and pituicytes allows them to retain a latent capacity for self-renewal, as documented for brain astrocytes (Bani-Yaghoub et al., 2006).

In conclusion, we provide here compelling evidence that both the posterior pituitary pituicytes and the anterior pituitary FSCs are astroglial cells specialized to serve pituitary cell functions. Distinct postnatal pathways for FSC and pituicyte regeneration do not exclude the possibility that both cell types have a common developmental origin, but from a transcriptomic perspective, pituicyte maturation separates them postnatally. We further show that FSCs comprise at least two subtypes of specialized cells, with FS1 equipped for classical FSC functions and FS2 equipped for stem cell functions. Such a division of function resembles the neurogenic regions of the brain, the subventricular zone and the dentate gyrus, where typical astrocytes coexist with a subpopulation of radial glial-like neural stem cells that express astroglial and stem cell markers (Berg et al., 2018; Zywitza et al., 2018).

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DATA AVAILABILITY STATEMENT
The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE184319 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184319).

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