The Adult Pituitary Contains a Cell Population Displaying Stem/Progenitor Cell and Early Embryonic Characteristics

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A side population (SP) has been identified in a number of tissues, where it typically represents a small population enriched in stem/progenitor cells. In this study we show that the adult mouse anterior pituitary (AP) also contains a characteristic SP displaying verapamil-sensitive Hoechst dye efflux capacity. A majority of the SP cells express stem cell antigen 1 at a high level (Sca1<sup>high</sup>). Using (semi)quantitative RT-PCR and immunofluorescence, we characterized the Sca1<sup>high</sup> SP as a population enriched in cells expressing stem/progenitor cell-associated factors and components of the Notch, Wnt, and sonic hedgehog signaling pathways, functional in stem cell homeostasis as well as in early pituitary embryogenesis. Lhx4, a transcription factor pivotal for early embryonic development, was only detected in the Sca1<sup>high</sup> SP, whereas Lhx3, in contrast to Lhx4 not down-regulated after AP development, was only found in the main population. The Sca1<sup>high</sup> SP was depleted from cells expressing phenotypic markers of differentiated AP cells (hormones), but contained a small proportion of folliculo-stellate cells. Stem cells of many tissues can clonally expand to nonadherent spheres in culture. Clonal spheres also developed in AP cell cultures. Spheres showed an expression pattern resembling that of Sca1<sup>high</sup> SP cells. Moreover, the sphere-initiating cells of the pituitary segregated to the SP and not to the main population. In conclusion, we show that the adult pituitary contains a hitherto undescribed population of cells with SP phenotype and clonal expansion capacity. These cells express (signaling) molecules generally found in stem/progenitor cells and/or operative during pituitary early embryonic development. These characteristics are supportive of a stem/progenitor cell phenotype. (Endocrinology 146: 3985–3998, 2005)

The Anterior Pituitary (AP) is an endocrine organ that dynamically adjusts its hormone output to changing physiological and pathological conditions. Some of these adaptations are associated with rapid expansion of specific AP cell populations. It is unknown whether stem/progenitor cells play a role in this expansion. On the other hand, the cellular constitution of the mature gland appears stable under basal conditions, implying steady cell turnover, which is estimated to be approximately 1.5%/d in the young adult male rat (1). Formation of new hormone-producing cells is facilitated by a population enriched in cells expressing phenotypic markers of differentiated AP cells (hormones), but contained a small proportion of folliculo-stellate cells. Stem cells of many tissues can clonally expand to nonadherent spheres in culture. Clonal spheres also developed in AP cell cultures. Spheres showed an expression pattern resembling that of Sca1<sup>high</sup> SP cells. Moreover, the sphere-initiating cells of the pituitary segregated to the SP and not to the main population. In conclusion, we show that the adult pituitary contains a hitherto undescribed population of cells with SP phenotype and clonal expansion capacity. These cells express (signaling) molecules generally found in stem/progenitor cells and/or operative during pituitary early embryonic development. These characteristics are supportive of a stem/progenitor cell phenotype. (Endocrinology 146: 3985–3998, 2005)

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Abbreviations: AMCA, Amino-methylcoumarin acetic acid; AP, anterior pituitary; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; DAPl, 4',6-diamidino-2-phenylindole; E, embryonic day; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; ES, embryonic stem; FACS, fluorescence-activated cell sorter; FS, folliculo-stellate; Fzd, frizzled; gGUS, glycoprotein hormone α-subunit; Hes1, hairy and enhancer of split 1; HSC, hemopoietic stem cell; M-MuLV, Moloney murine leukemia virus; MP, main population; NSC, neural stem cell; PE, phycoerythrin; PI, propidium iodide; POMC, pro-opiomelanocortin; PRL, prolactin; Pth1, Patched 1; RNase, ribonuclease; Sca1, stem cell antigen 1; Shh, sonic hedgehog; SP, side population; WT, wild type.

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characterize potential stem/progenitor cells from a number of other tissues (retina, skin, mammary gland, heart, and pancreas) (14–18).

Despite the lack of universal stem cell markers, certain cellular molecules have been found in at least part of the stem cells isolated, such as stem cell antigen 1 (Sca1), c-Kit (CD117), CD34, prominin-1 (CD133/AC133), Oct-4 (also called Oct-3, NF-A3, or Pou5f1), Nanog, nestin, and Bmi-1 (7, 8, 11, 14, 15, 17–19–27). These factors can be considered prospective identifiers of candidate stem/progenitor cells. In addition to these cellular markers, the signaling pathways of Notch, Wnt, and sonic hedgehog (Shh) have recently been implicated in stem cell renewal and fate determination in a variety of tissues (brain, intestine, bone marrow, mammary gland) (28–36).

Cues for adult stem cell characterization may also be gathered from embryonic development (19, 31, 34). The AP develops from Rathke’s pouch that starts to invaginate from the oral ectoderm on embryonic d 8.5 (E8.5) in the mouse (37). Morphogenesis and maturation of the gland are driven by an orchestrated action of extrinsic and intrinsic factors. Gradients of these factors leave a locally and temporally precise imprint of transcription factor expression, instrumental for cell type determination and expansion (37, 38). Among those signals, Notch, Wnt, and Shh appear to be essential (37, 39, 40). Furthermore, the LIM homeobox transcription factors, Lhx3 (P-Lim) and Lhx4 (Gsh-4), play a crucial role in lineage specification and expansion of AP cell types during early embryonic development (41, 42). Lhx3 expression persists into adulthood, whereas the expression of Lhx4 is significantly down-regulated from E15.5, coinciding with the period of terminal differentiation of AP cells (41–43).

In the present study we searched for potential stem/progenitor cells in the adult AP on the basis of dye efflux and sphere-forming capacity. We identified a population of cells with SP phenotype that clonally replicate to nonadherent spheres and express candidate stem/progenitor cell markers, signaling molecules known to be involved in stem cell renewal and fate decision, and pituitary-related embryonic signals and transcription factors.

Materials and Methods

Materials

DMEM/F12, B27, N2 supplement, and chemically defined culture medium (44, 49) were produced by Invitrogen Life Technologies, Inc. (Grand Island, NY), and BSA by Serva (Heidelberg, Germany). Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were obtained from R&D Systems (Minneapolis, MN). Trypsin (from porcine pancreas) was purchased from ICN Biomedicals (Aurora, OH); Hoechst 33342 (bisbenzimide), verapamil, and propidium iodide (PI) from Sigma–Aldrich Corp. (St. Louis, MO); and bromodeoxyuridine (BrdU) from BD Biosciences (San Jose, CA).

All PCR reagents were supplied by Applied Biosystems (Applera Belgium, Lennik, Belgium). Ribonuclease (RNase) inhibitor (RNasin) was purchased from Promega Corp. (Leiden, The Netherlands), and Moloney murine leukemia virus (MuLV) reverse transcriptase, RNase-free deoxyribonuclease I (amplification grade), and oligonucleotide primers were obtained from Invitrogen Life Technologies, Inc. TaqMan RNA isolation reagent was purchased from Roche (Brussels, Belgium), and RiboGreen quantitation reagent from Molecular Probes (Eugene, OR). TaqMan Universal PCR Mix was obtained from Applied Biosystems; MasterMix Plus for SYBR Green I and TaqMan probes (designed using PrimerXpress, Applied Biosystems) were obtained from Eurogentec (Seraing, Belgium). Quantitative real-time PCR was run on an ABI PRISM 7700 or ABI PRISM 7000 Sequence Detector (Applied Biosystems).

The dipeptide β-Ala-Lys-Ne-amino-methylcoumarin acetic acid (AMCA) was provided by Dr. K. Bauer (Max Planck Institute, Hannover, Germany). Antibodies used for flow cytometry were R-phycocerythrin (R-PE)-conjugated rat antimouse Sca1, R-PE- and PE-Cy5-conjugated rat antimouse CD45, R-PE- and fluorescein isothiocyanate-conjugated rat antimouse CD117 (c-Kit), and R-PE-conjugated rat antimouse CD34 as well as the corresponding isotype controls (all from BD Biosciences). Mouse anti-BrdU antibody was obtained from BD Biosciences. Antibodies for immunofluorescent analysis of sorted cells included goat anti-Notch1 (Notch1 C-20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit antisera against Lhx3 and Lhx4 (provided by Dr. S. Pfaff, The Salk Institute for Biological Studies, La Jolla, CA); mouse antinestin (Rat-401) from BD Biosciences; rabbit antinestin (provided by Dr. R. McKay, National Institutes of Health, National Institute for Neurological Disorders and Stroke, Bethesda, MD); and antiserum against pituitary hormones (provided by Dr. A. F. Farlow through the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA). The secondary antibodies, Alexa Fluor 555-labeled goat antimouse and Alexa Fluor 488-labeled goat antirabbit, as well as the nuclear stain, ToPro-3, were purchased from Molecular Probes. Cy3-conjugated donkey anti-goat was obtained from Jackson ImmunoResearch Laboratories, Inc. (Soham, UK), and 4′-diamidino-2-phenylindole (DAPI) was purchased from Sigma–Aldrich Corp. The mounting medium, Vectashield, was provided by Vector Laboratories, Inc. (Burlingame, CA).

Animals

FVB mice (8-wk-old female; 3-wk-old male and female) were purchased from Elevage Janvier (Schaik, The Netherlands). Green fluorescent protein (GFP) transgenic mice that ubiquitously express enhanced GFP (eGFP) were provided by Dr. A. Nagy (Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, Canada), and backcrossed to the FVB genetic background for more than 20 generations. All animals were kept in an environment of constant temperature, humidity, and day-night cycle within the animal house facilities and had free access to water and food (University of Leuven, Leuven, Belgium). All animal experiments were conducted in accordance with the Endocrine Society’s ethical guideline, and were approved by the University of Leuven ethical committee.

Isolation of anterior pituitary cells

Mice were killed by CO₂ asphyxiation, followed by decapitation. For SP analysis and sorting, animals were first perfused with PBS to rinse blood out of the pituitary. The neurointermediate lobe was carefully discarded under the stereomicroscope. Anterior lobes (AP) were dissociated into single cells using trypsin (2.5%; 15 min) as previously described in detail (44). Cells were finally resuspended in serum-free, chemically defined culture medium (45).

Flow cytometric analysis of AP cells after incubation with Hoechst 33342 and membrane antigen staining

We adapted the original SP identification method of Goodell et al. (6) for optimal resolution of pituitary SP cells. The main difference was the medium used (chemically defined culture medium instead of DMEM). Cell density was adjusted to 1 × 10⁶ cells/ml. Hoechst 33342 was added to a final concentration of 2.5 μg/ml together with verapamil (50–100 μM) to avoid control samples to vary because of the blockage of the nuclear immunofluorescence of the SP phenotype. Samples were incubated for 90 min at 37° C, then for 20–30 min on ice with R-PE-labeled anti-Sca1, R-PE- or fluorescein isothiocyanate-labeled anti-c-Kit, R-PE-labeled anti-CD34, and/or R-PE- or PE-Cy5-labeled anti-CD45 (all at 1 μg/10⁶ cells). Cells were finally resuspended in ice-cold PBS with fetal calf serum (2%) and PI (2 μg/ml) for fluorescence-activated cell sorter (FACS) analysis and sorting. CD45 expression within the Sca1⁹ SP was analyzed by flow cytometry of Hoechst-incubated cells stained with anti-Sca1-R-PE and anti-CD45-PE-Cy5 (analyzed in three independent cell isolations). To control whether trypsinization affected membrane antigen staining, mouse bone marrow

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cells were treated with trypsin in an identical way as AP cells and stained for Sca1, CD45, c-Kit, and CD34. FACS analysis revealed no differences between untreated and trypsin-treated bone marrow cells (data not shown).

Flow cytometric analysis was carried out with a FACSVerse (BD Biosciences). The SP was visualized using dual-wavelength flow cytometry with UV excitation and detection of blue emission with a BP 424/44 filter and of red emission with a BP 630/22 filter, resolving the total population in a Hoechstdiluent SP and a Hoechstmixed high SP. Cells were analyzed and sorted within the living (PI-) cell population.

In some experiments the dipeptide β-Ala-Lys-Ne-AMCA (100 μM) was added to the cells during incubation with Hoechst as a marker for folliculo-stellate (FS) cells (46). Cells were analyzed by FACS for SP phenotype (Hoechst exclusion) and FS cell phenotype (dipeptide uptake) using UV excitation.

**Immunofluorescence analysis of SP and main population (MP) cells sorted by FACS**

SP and MP cells were sorted by FACS in chemically defined medium and deposited by cytopsin on glass slides. Cells were permeabilized with Triton X-100 (0.4% in PBS), blocked with γ-Heptadecyl glucoside (10% in PBS) and incubated with primary antibodies (rabbit antinestin at 1:200; mouse anti-nestin at 1:100; anti-Notch1 at 1:100; anti-Lhx3 at 1:1000; anti-Lhx4 at 1:500; hormone antisera at the dilutions recommended by Dr. A. F. Parlow) for 2 h, and finally with secondary antibody (Alexa-labeled antiserum at 1:1000; Cy3-labeled antibody at 1:400) for 1.5 h. Cells were covered with Vectashield and analyzed using an epifluorescence microscope (DMRB, Leica, Wetlitz, Germany). Negative controls were performed by omitting primary antibodies and substituting the primary antibodies with mouse nonsense IgG or normal serum at comparable dilutions.

**Growth and characterization of nonadherent cells from AP spheres**

Mouse AP cells were prepared as described above and seeded in 35-mm nontreated petri dishes at a density of 100,000 cells/ml (2 ml/dish) in either DMEM/Ham’s F-12 or chemically defined culture medium, both supplemented with B27 (1:50) and bFGF (20 ng/ml); in some experiments replaced by EGF at 20 ng/ml). Cells were kept at 37 °C in a 1.5% CO2 incubator. From d 7–8 in culture, clumps and cells in suspension started to stick to the spheres to eventually form larger aggregates. Spheres could be recovered from these aggregates by treating them at 37 °C for 5 min with trypsin-EDTA (0.05% trypsin in DMEM with 0.037% EDTA, 0.3% BSA, and anorganic salts).

To explore the potential for self-renewal, primary spheres were dispersed into single cells, and generation of secondary spheres was examined with Vybrant dyes, and spheres were picked from cultured cells incubated with trypsin-EDTA for 15 min at 37 °C, and then mechanically dispersed under a pancreatic microptic control. Trypsin was inhibited by adding the same volume of 10% fetal calf serum (diluted in chemically defined medium). Cells were spun down immediately, resuspended in sphere culture medium, analyzed for single cellularity, and cultured at 10,000 cells/ml (2 ml/dish) for secondary sphere generation.

To study whether pituitary spheres were clonal in origin, sphere cultures were initiated as described above from a 1:1 mixture of wild-type (WT) and GFP° AP cells isolated from FVB WT and FVB eGFP expressing transgenic mice, respectively. At different time points in culture, all floating material in a dish (spheres, aggregates, irregular cell clumps, and single cells in suspension) was fixed with formaldehyde (4% in PBS) for 15 min at room temperature. Samples were mounted in Vectashield for epifluorescence analysis or for examination using a confocal laser scanning microscope (LSM 510, Zeiss, Zaventem, Belgium). Nuclear counterstain was obtained with DAPI for epifluorescence analysis and with ToPro-3 for confocal analysis.

To examine whether spheres developed by cell division as opposed to cellular aggregation, the thymidine analog BrdU (10 μM) was added to the cells 1 d after seeding. On d 3, all cell material in suspension was fixed as described above and further analyzed for BrdU incorporation by immunofluorescent staining. Therefore, cells were permeabilized with 0.5% saponin in PBS and treated with deoxyribonuclease I (100 U/ml) for 30 min at 37 °C for BrdU antigen retrieval. Then the cell pool was successively incubated with normal goat serum (20%; 10 min), anti-BrdU antibody (1:2.5; overnight), and Alexa Fluor 550 goat antimouse (1:1000; 1.5 h). Samples were mounted in Vectashield.

For analysis of protein expression by immunofluorescence, spheres and clumps were treated as described for BrdU immunostaining, with omission of the antigen retrieval step, using the antibody dilutions mentioned above.

The number of cells per putitary sphere was counted as ToPro-3-stained nuclei in images of consecutive confocal slices and as DAPI-labeled nuclei in epifluorescent images. Diameters were measured on live culture pictures using analysis tools provided by ImageJ (freely available at http://rsb.info.nih.gov/ij/).

**Gene expression analysis by RT-PCR**

RT. Sca1high AP cells and MP cells within the living (PI-) cell population were sorted by FACS in TriPure with glycogen. A typical yield of Sca1high SP cells from a single sort was between 20,000 and 50,000 cells. In some experiments, cells outside both the SP and MP gates (non-SP+non-MP) were also sorted (see Fig. 1A for sorting gates). Total RNA was isolated according to the manufacturer’s recommendations and dissolved in 5 μl RNase-free H2O. A comparable amount of RNA was obtained from an identical number of SP and MP cells. The RNA solution was treated with RNase-free deoxyribonuclease I (1 U), quantified with RiboGreen quantitation reagent following the manufacturer’s protocol (Molecular Probes), and stored at −80 °C until additional analysis by RT-PCR.

Pituitary spheres or irregular groups of cells (clumps) of comparable size were individually picked from cultures, rinsed in PBS (37 °C), and transferred to PCR tubes into 10.2 μl RNase-free H2O for omotic lysis. The samples were heated at 65 °C for 10 min and stored at −80 °C until additional analysis by RT-PCR. To control the reliability of the single sphere or clump lysis method for gene expression analysis, RT-PCR was also performed on RNA isolated from an identical number of cells derived from dissociated spheres or clumps. Spheres and clumps were picked to be pooled within their respective group and were dispersed into single cells as described above. RNA was isolated from 10,000 cells of each (a comparable amount was obtained from this same number of sphere and clump cells) and checked by semiquantitative RT-PCR as described below.

For RT, 2 μl RNA solution from Sca1high SP cells, from MP or from non-SP+non-MP cells was added to 18 μl of the following RT reaction mixture: 4 μl MgCl2 (25 mm), 2 μl 10× PCR Gold buffer, 0.8 μl dNTPs (100 mm), 1 μl random hexamers (50 μm), 1 μl M-MuLV reverse transcriptase (200 U/μl), 1 μl RNasin (40 U/μl), and 8.2 μl RNase-free H2O. For RT of clump or sphere RNA, 9.8 μl RT mixture (without H2O) was added to the lysed cell material. RT was performed using the following temperature cycle: 10–15 min at 25 °C, 50 min at 42 °C, and 10 min at 95 °C. As a positive control for the RT reaction, 10 ng total RNA, purified from perfused mouse AP, were added to the RT mixture. As a negative control, RNase-free H2O was used.

PCR. PCR was performed on 1 μl of the RT reaction, added to 9 μl of a PCR mixture containing 0.9 μl MgCl2 (25 mm); except for Oct-4 amplification, where 0.75 μl was optimal, 1.1 μl 10× PCR Gold buffer, 0.1 μl dNTPs (100 mm), 0.15 μl AmpliTaq Gold DNA polymerase (5 U/μl), 6.55 μl RNase-free H2O, and 0.1 μl sense and antisense oligonucleotide primers (100 μM each). The nucleotide sequences of the PCR primers are presented in Table 1. Primers were designed using Vector NTI (InforMax, Invitrogen Life Technologies, Inc.) to span at least one intron except for frizzled (Fzd), which lacks introns. To control for amplification of the Fzd gene from residual genomic DNA due to incomplete DNA digestion, PCR on an RT sample run without M-MuLV was performed. The expression of Fzd receptors was analyzed using PCR primers in consensus sequences of all known Fzd receptor subtypes (Fzd1–10; primers were called FzdAll; see Table 1). RT-PCR for L19, a ribosomal protein constitutively expressed in all cells, was used as an internal control. No L19-negative sphere or clump samples were observed. PCR conditions were optimized using total RNA from perfused mouse APs, and the final specific parameters used are also detailed in Table 1. The reproducibility of the PCR was 80–100% as tested on 10 spheres and clumps in five independent PCRs. The reproducibility of the PCR for hormones has been determined previously as being at least 80% (47).
For semiquantitative RT-PCR, the cycle number was varied between 35 and 50, as explained below. The identities of the amplified fragments were confirmed by sequence analysis (performed by Lark Technologies, Essex, UK). PCR protocols for hormones have been optimized and validated previously in the laboratory (47). In general, PCR cycling consisted of the following temperature steps: 7 min at 95 C, followed by 35–50 cycles of denaturation, annealing, and extension, the details of which depend on the specific cDNA to be amplified (described in Table 1). Finally, samples were heated at 72 C for 7 min before cooling to 4 C. A positive control sample for PCR performance consisting of 10 ng reverse transcribed mouse AP total RNA was included as well as a negative control consisting of 1 µl RNase-free H2O instead of sample. Spheres and clumps to be compared (from the same culture) were simultaneously subjected to RT using the same RT mixture, and RNA dilution series of Sca1high SP and MP (and in some experiments non-SP) were first checked on the basis of L19 mRNA quantitation are given in Table 1. Real-time RT-PCR for glycoprotein hormone α-subunit (αGSU) and markers was carried out in an analogous way with SYBR Green as quantifying reagent, using MasterMix Plus for SYBR Green I instead of TaqMan Universal PCR Mix. The specificity of the amplification reactions quantified with SYBR Green was verified by melt curve analysis. Each sample was measured in triplicate. Data were analyzed with SDS software (Applied Biosystems).  

Quantitative real-time RT-PCR. To inspect the reliability of the semiquantitative method, quantitative real-time RT-PCR was performed for a selection of the markers using the primers shown in Table 1 or other primers already published (47). As an additional control, another internal (normalization) standard (18S rRNA instead of L19 mRNA) was used. 18S rRNA levels were comparable in an identical amount of Sca1high SP and MP total RNA. Real-time RT-PCR for hormones was performed using sequence-specific TaqMan probes as described in detail previously (47); sequences used for proopiomelanocortin (POMC) mRNA quantitation are given in Table 1. Real-time RT-PCR for glycoprotein hormone α-subunit (αGSU) and markers was carried out in an analogous way with SYBR Green as quantifying reagent, using MasterMix Plus for SYBR Green I instead of TaqMan Universal PCR Mix. The specificity of the amplification reactions quantified with SYBR Green was verified by melt curve analysis. Each sample was measured in triplicate. Data were analyzed with SDS software (Applied Biosystems). Relative expression values were obtained using the ∆ threshold cycle/standard curve method and were normalized to (relative) 18S rRNA levels as described previously (47). The normalized expression values were used to calculate the SP/MP expression ratios.

Fig. 1. The adult mouse AP contains a SP enriched in Sca1high-expressing cells. Dual-wavelength FACS analysis reveals the presence of typical SP cells (1.7% of total living cells) in the adult AP that are Hoechstlow because of efficient Hoechst 33342 efflux capacity (A) that can be blocked by verapamil (B). The SP contains cells with high expression of Sca1 (C; only cells gated as SP in A are shown). A large proportion of the SP cells (70%) are Sca1high, whereas the majority of the residual non-SP cells are Sca1low (D; mean ± SEM of 13 independent cell isolations are shown).
### TABLE 1. Oligonucleotide primers, cycling parameters, and expected amplicon sizes for PCR amplification of the markers analyzed

<table>
<thead>
<tr>
<th>Marker</th>
<th>PCR oligonucleotide primers</th>
<th>GenBank accession no. (gi)</th>
<th>Length(s) of denaturation annealing and elongation steps; cycle no.</th>
<th>Annealing temp. (C)</th>
<th>Size of amplified fragment (bp)</th>
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<td>Oct-4</td>
<td><strong>S</strong>:5’-CAAGTGGCAGCTGAGACTTGGC-3’</td>
<td>7305398</td>
<td>10; 20; 25; 45</td>
<td>56</td>
<td>299</td>
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<td></td>
<td><strong>AS</strong>:5’-TCAGAAGGACAGAATGCTGAC-3’</td>
<td>(290–581)</td>
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<td>Nanog</td>
<td><strong>S</strong>:5’-TTCTGCTGGCTGTCAGGGAT-3’</td>
<td>20931594</td>
<td>10; 20; 35; 45</td>
<td>63</td>
<td>238</td>
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<tr>
<td></td>
<td><strong>AS</strong>:5’-CTGCTGGCAGGCTCTTGAGAT-3’</td>
<td>(533–770)</td>
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<td>Nestin</td>
<td><strong>S</strong>:5’-AACGACGAGGCTCAGAGTCTAC-3’</td>
<td>15011580</td>
<td>10; 20; 25; 40</td>
<td>58.4</td>
<td>299</td>
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<td>Prom1</td>
<td><strong>S</strong>:5’-GTACCTGAGGCTGAGGAGGAC-3’</td>
<td>2789657</td>
<td>10; 20; 25; 45</td>
<td>63.5</td>
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<td>(1158–1526)</td>
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<td>Bmi-1</td>
<td><strong>S</strong>:5’-TGTCGGATCTGAGCTGCTGAG-3’</td>
<td>46849765</td>
<td>10; 15; 25; ND</td>
<td>57</td>
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<tr>
<td></td>
<td><strong>AS</strong>:5’-CAAGATGGTGGACAGCACA-3’</td>
<td>(24–295)</td>
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<td>Lhx3</td>
<td><strong>S</strong>:5’-CTCGTCTGGAGGGCTGCTC-3’</td>
<td>6754539</td>
<td>10; 20; 35; 40</td>
<td>60</td>
<td>350</td>
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<tr>
<td></td>
<td><strong>AS</strong>:5’-TCTCCTCTGCTGAGGGCTC-3’</td>
<td>(462–811)</td>
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<tr>
<td>Prop1</td>
<td><strong>S</strong>:5’-TGCCCTTGGACTGGCTGAC-3’</td>
<td>6679480</td>
<td>10; 20; 25; 50</td>
<td>59.3</td>
<td>312</td>
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<td></td>
<td><strong>AS</strong>:5’-GCTGAGGAAATGCGCTCTT-3’</td>
<td>(308–619)</td>
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<tr>
<td>SI100(β)</td>
<td><strong>S</strong>:5’-ATGCGTCCGAGCTGGAAGAC-3’</td>
<td>54262155</td>
<td>10; 15; 25; 50</td>
<td>63</td>
<td>188</td>
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<tr>
<td></td>
<td><strong>AS</strong>:5’-CTCGCTCGGTCCGTCTCACA-3’</td>
<td>(115–302)</td>
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<td>GH</td>
<td><strong>S</strong>:5’-TGGGCTCAAGGAGGATGGCTTCT-3’</td>
<td>51067</td>
<td>15; 20; 45; 35–40</td>
<td>60</td>
<td>307</td>
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<td></td>
<td><strong>AS</strong>:5’-AAATCTGCTGGAGGAGCTGACG-3’</td>
<td>(119–425)</td>
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<td>PRL</td>
<td><strong>S</strong>:5’-ACCAGAGAGGGACGCTG-3’</td>
<td>53783</td>
<td>15; 20; 45; 35–40</td>
<td>58</td>
<td>281</td>
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<td><strong>AS</strong>:5’-GCTGAGGAAATGCGCTCTT-3’</td>
<td>(52–332)</td>
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<td>POMC</td>
<td><strong>S</strong>:5’-CGAGATTCTTGCTAGCTGCTCAG-3’</td>
<td>200439</td>
<td>15; 20; 45; 40</td>
<td>60</td>
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<td><strong>AS</strong>:5’-TCTGAGCAGCGGGTGAGGAC-3’</td>
<td>(117–139)</td>
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<td><strong>TM-S</strong>:5’-AGGCGAGGCGCTGAGGAC-3’</td>
<td>200440</td>
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<td><strong>TM-P</strong>:5’-AGGCGAGGCGGGTGAGGAC-3’</td>
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<td>202179</td>
<td>15; 15; 20; 35–40</td>
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<td><strong>AS</strong>:5’-AGGAGATAGCTGCTGACG-3’</td>
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<td>LHβ</td>
<td><strong>S</strong>:5’-TCTCTCTGCTGAGGAAATGCG-3’</td>
<td>1772991</td>
<td>15; 20; 30; 35–40</td>
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<td><strong>AS</strong>:5’-CAATGGTCAGGCTGACG-3’</td>
<td>(78–274)</td>
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<td>FSHβ</td>
<td><strong>S</strong>:5’-CTTCTCAGTGGCTCTGAGGAAATGCG-3’</td>
<td>40254640</td>
<td>15, 15, 40; ND</td>
<td>55.5</td>
<td>300</td>
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<td></td>
<td><strong>AS</strong>:5’-CAATGGTCAGGCTGACG-3’</td>
<td>(81–380)</td>
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<td>TSHβ</td>
<td><strong>S</strong>:5’-CTTCTCAGTGGCTCTGAGGAAATGCG-3’</td>
<td>202182</td>
<td>15, 15, 40; ND</td>
<td>55.5</td>
<td>280</td>
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<td></td>
<td><strong>AS</strong>:5’-AGGCGAGGCGGGTGAGGAC-3’</td>
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<td>L19</td>
<td><strong>S</strong>:5’-AGAGATAGCTGCTGACG-3’</td>
<td>198642</td>
<td>15; 20; 45; 40</td>
<td>59</td>
<td>311</td>
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<td><strong>AS</strong>:5’-AGGCGAGGCGGGTGAGGAC-3’</td>
<td>(58–368)</td>
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</table>

**a** Sense primer; **AS**, antisense primer; **TM-S**, TaqMan sense primer; **TM-AS**, TaqMan antisense primer; **TM-P**, TaqMan probe.

**b** Underlined are consensus regions of Fzd1–10; position of amplified fragment in GenBank entry of Fzd4 is between consensus regions of S and AS.

**c** No GenBank entry with complete CDS available; positions of S and AS (and not of amplified fragment) are given.

**d** Cycle number used for PCR of sphere and clump samples is in **boldface** text; for semiquantitative PCR, cycle number was varied (35, 40, 45 and 50 cycles); ND, Not determined in spheres and clumps.
Results

Pituitary contains cells with SP phenotype

AP cells from perfused adult mice were stained with Hoechst 33342 and analyzed by dual-wavelength flow cytometry. A Hoechstlow SP was detected, comprising 1.7% (mean ± SEM, 1.74 ± 0.05%; n = 14 independent cell isolations) of the total number of AP cells (Fig. 1A). The SP phenotype was confirmed by the disappearance of the Hoechstlow cell population when verapamil was added during incubation with Hoechst (Fig. 1B), as has been shown for the SP of other tissues (6–11). Cytometric analysis also revealed that pituitary SP cells were cells of small to medium size and of very low granularity, clearly different from the bulk of AP cells. SP cells were also identified in AP from immature (3 wk old) mice (2.39 ± 0.08% of total cells; n = 5) as well as in immature (2 wk old) and adult, rat, and chicken pituitaries (data not shown).

Adult pituitary SP is enriched in cells expressing Sca1, but not c-Kit or CD34

SP cells from perfused adult AP were characterized by flow cytometry for the expression of HSC markers reported to be present on stem/progenitor cells and SP cells from other tissues (7, 8, 11, 17, 19). A majority (70.87 ± 1.33%; n = 13) of the adult SP cells expressed Sca1 at a high level (Sca1high SP cells), whereas the remaining SP cells were Sca1 negative (Sca1neg; Fig. 1, C and D). In the non-SP cell population, the high majority of cells were Sca1neg; 5% (5.20 ± 0.86%) expressed Sca1 at a low level (Sca1low), and 2.5% (2.42 ± 0.26%) at a high level (Fig. 1D). Neither the expression of c-Kit nor that of CD34 (a HSC marker in man) was detected in SP cells (n = 3). The pan-hemopoietic marker CD45 was present on 5% of the SP cells (5.09 ± 0.40%; n = 4) and on 1% (1.01 ± 0.29%) of the non-SP cells. However, no CD45 expression was detected within the Sca1high SP, as analyzed by flow cytometry of Hoechst-incubated cells stained with anti-Sca1-R-PE and anti-CD45-PE-Cy5 (n = 3), thereby excluding the presence of HSC in the pituitary Sca1high SP.

Epitopes of the membrane antigens CD45, CD34, c-Kit, and Sca1 were not destroyed by trypsin treatment as tested in bone marrow (see Materials and Methods).

Fig. 2. The adult AP Sca1high SP is enriched in cells expressing mRNA of stem/progenitor cell- and embryonic pituitary-related factors and signaling molecules. Semiquantitative RT-PCR was performed to compare mRNA expression levels in FACS-sorted Sca1high SP and MP. A, A representative example is shown. A dilution series of Sca1high SP and MP RNA (from 10^4–10^0 pg/µl), showing comparable L19 expression, was analyzed by RT-PCR for Notch1 expression. In Sca1high SP cells, Notch1 mRNA can be detected in a 100-fold lower RNA amount than in MP cells, resulting in a Notch1 mRNA SP/MP expression ratio of 100. In addition to varying the input amount of RNA, amplification cycle numbers were varied (not shown) to ensure quantification in exponential phase (see Materials and Methods). B and C, Representative PCR signals obtained under exponential amplification conditions are shown together with the final SP/MP expression ratio. For each factor analyzed, RNA isolated from three to five independent cell sortings was checked by RT-PCR, performed one or two times per cell sort. Higher mRNA levels of stem/progenitor cell-associated factors as well as of members of signaling systems (Notch, Notch1, and Hes1; Wnt, Fzd8; Shh, Ptc1), operative in stem cell regulation and during pituitary embryogenesis, are found in SP than in MP (B), except for Fzd2. With respect to pituitary-related factors (C), markers of mature pituitary cell phenotypes (hormones) are expressed at a higher mRNA level in MP than in SP. The FS cell marker S100 was only detected in SP (and in the residual non-SP–non-MP; data not shown). Lhx4, which is crucial for early embryonic pituitary development, is only detected in SP, whereas Lhx3, which, in contrast to Lhx4, is not down-regulated after development, is only found in MP. D, Quantitative real-time RT-PCR was performed to verify the results obtained by semiquantitative RT-PCR. Similar differences in expression between SP and MP were found. The means of two independent measurements (two independent cell sortings, each analyzed once) are shown. > and < indicate that no amplification signal is detected in MP or SP, respectively, even at the maximum conditions used (10^4 pg/µl RNA and 50 cycles of PCR amplification). M, 100-bp marker; Prom1, prominin-1.
**Adult AP Sca1<sup>high</sup> SP is enriched in cells expressing stem/progenitor cell-associated factors**

Sca1<sup>high</sup> (CD45<sup>neg</sup>) SP cells as well as MP cells (see Fig. 1A) were sorted by FACS and analyzed by semiquantitative RT-PCR (as an example, see Fig. 2A) for the expression of genes associated with a stem/progenitor cell phenotype in other tissues, *i.e.* Oct-4 and Nanog expressed in stem cells of the embryo (ES cells), and nestin, prominin-1 and Bmi-1, mainly found in NSC and HSC (14, 15, 20–27). Results are shown in Fig. 2B. Oct-4 and Nanog mRNA were detected in SP and were not detectable in MP, at least under the conditions used (for amounts of RNA and cycle numbers, see Materials and Methods). Semiquantitative RT-PCR revealed a 10-fold higher level of nestin, Bmi-1, and prominin-1 mRNA in SP than in MP. Quantitative real-time RT-PCR confirmed these higher expression levels in SP (Fig. 2D). In accordance, nestin protein expression was predominantly detected in SP cells, as analyzed by immunofluorescence on sorted SP and MP cells (Fig. 3, A and B).

**Adult AP Sca1<sup>high</sup> SP is enriched in cells expressing members of Notch, Shh, and Wnt signaling pathways**

Sca1<sup>high</sup> (CD45<sup>neg</sup>) SP cells and MP cells were analyzed by RT-PCR for the expression of signaling molecules that are functional in stem cell renewal and fate determination and that also play a key role during pituitary early embryonic development (28–37, 39, 40). Semiquantitative RT-PCR (Fig. 2B) showed 100-fold higher levels of Notch1 mRNA in SP than in MP, and 100- to 1000-fold higher mRNA values of its downstream target hairy and enhancer of split 1 (Hes1). The mRNA of the Shh receptor Patched1 (Ptch1; also referred to as Ptc1) was 10-fold more abundant in SP than in MP. Expression levels of Notch1, Hes1, and Ptch1 were confirmed by quantitative real-time RT-PCR; SP/MP ratios were of the same order of magnitude as those obtained by semiquantitative RT-PCR (see Fig. 2D: SP/MP = 392 for Notch1, 694 for Hes1, and 7 for Ptch1). Similarly, Notch1 protein was present at a detectable level in (some) cells of the SP, but not in cells of the MP (Fig. 3, C and D).

The expression of frizzled (Fzd) receptors, implied in canonical Wnt signaling, was analyzed by RT-PCR, first using PCR primers in consensus sequences of all known Fzd receptor subtypes (Fzd1–10; see Table 1). A 10-fold higher level of Fzd mRNA expression (FzdAll) was detected in SP vs. MP (Fig. 2B). Sequencing of the amplified fragments revealed predominant amplification of Fzd1 and Fzd2 from MP, and of Fzd8 from SP. Using sequence-specific PCR primers (see Table 1), it was found that Fzd8 mRNA was 10-fold higher in SP than in MP, whereas Fzd2 mRNA showed the opposite picture. Quantitative real-time RT-PCR supported the differences obtained by semiquantitative RT-PCR (Fig. 2D).

**Adult AP Sca1<sup>high</sup> SP is enriched in cells expressing the early embryonic pituitary transcription factor Lhx4, but not Lhx3 or hormones**

Sca1<sup>high</sup> SP cells as well as MP cells were analyzed by RT-PCR for the expression of transcription factors essential during early embryonic development of the AP (37, 38, 41, 42) and of hormones as phenotypic markers of differentiated AP cells. Lhx4 was only detectable in SP, not in MP, at both the mRNA and protein levels (Fig. 2C and Fig. 3, E and F). In contrast, Lhx3 (which is not down-regulated after pituitary development) was only detected in MP (Fig. 2C and Fig. 3, G and H). Prop1, an embryonic transcription factor indispensable for development of the Pit-1 lineage (lactotropes, somatotropes, and thyrotropes) (49) was only found in MP (Fig. 2C). The αGSU mRNA level was 10-fold higher in MP than in SP, prolactin (PRL), LHβ, GH, and POMC levels were 100-fold higher, and FSHβ and TSHβ levels were more than 1000-fold higher (not detected in SP). Differences of the same order of magnitude were found using quantitative real-time RT-PCR (Fig. 2D). Correspondingly, PRL-immunoreactive...
cells were abundantly present in the MP, whereas they were depleted from the SP (Fig. 3, I and J).

**AP Sca^high^ SP contains cells with FS cell phenotype**

Sca^high^ SP and MP cells were analyzed by semiquantitative RT-PCR for the expression of S100 as a marker of AP FS cells. S100 mRNA was detected in SP, but not in MP (Fig. 2C). Because there are more FS cells (~7%) than SP cells (1.7%) in the adult mouse AP, we searched for the remainder of the FS cells. In addition to the SP and MP, the residual non-SP+non-MP cell population was sorted (see Fig. 1A for SP and MP sorting gates) and analyzed for S100 expression. S100 mRNA was found in the non-SP+non-MP at the same expression level as in the SP (data not shown). To analyze whether all or only part of the SP cells were FS cells, quantification of FS cells within the SP was performed by FACS analysis of AP cells simultaneously incubated with Hoechst and the dipeptide β-Ala-Lys-Ne-AMCA. Uptake of this dipeptide is a characteristic capacity of FS cells in the AP (46). FACS analysis revealed that dipeptide-positive cells accounted for 6% of the total AP cells (5.90 ± 0.35%; n = 4), a percentage comparable to the proportion of FS cells in the mouse AP (50). Within the SP, only 5.81 ± 0.50% (n = 4) of the cells were dipeptide positive.

**Nonadherent spheres develop in AP cell cultures under neurosphere-like growth conditions**

In a complementary approach to prospectively identify and characterize pituitary stem/progenitor cells, AP cells from 3-wk-old or adult mice were cultured under slightly modified neurosphere-forming conditions (see Materials and Methods). During stationary culture in serum-free, chemically defined culture medium (45) supplemented with B27 and bFGF, free-floating spheres progressively developed in time (Fig. 4). Spheres were bright, smooth-edged, and compact and were clearly different from irregular clumps of cells that also appeared in the culture (Fig. 4). On d 3 of culture (Fig. 4D), spheres displayed a diameter of 41.6 ± 1.0 μm (mean ± SEM of 39 spheres; range, 30.2–56.4 μm), and contained 17 ± 2 cells (mean ± SEM of 10 spheres; range, 9–26 cells). The spheres expanded in size as well as in number during the next 3 d. After 6 d in culture (Fig. 4F), 30–50 spheres were observed per 200,000 AP cells, displaying a diameter of 70.9 ± 5.7 μm (mean ± SEM of 10 spheres; range, 45.2–97.0 μm) and containing 42 ± 2 cells (mean ± SEM of 10 spheres; range, 32–54 cells). From d 6, spheres did not observably increase in size (Fig. 4, F to G), but clumps and cells in suspension started to attach to the spheres to eventually form larger aggregates (diameter, 100–300 μm; Fig. 4H). Spheres could be recovered from these aggregates by meticulously controlled trypsinization (see Materials and Methods). Spheres recovered from d 8–10 aggregates showed a diameter of 96.6 ± 4.6 μm (mean ± SEM of five spheres; range, 78.4–111.7 μm) and contained an estimated 100–200 cells.

Trials with other media were performed to grow pituitary spheres. B27 supplement provided essential components, which could not be replaced by N2, another supplement sometimes used to grow neurospheres. bFGF (or, alternatively, EGF) was found to be indispensable, and no spheres were obtained with other growth factors, such as nerve growth factor. When the chemically defined medium was replaced by the classical neurosphere culture medium DMEM/Ham’s F-12 (also supplemented with B27 and bFGF or EGF), floating spheres appeared, but a cavity almost immediately developed in the body of the sphere (data not shown). These hollow spheres transformed into aggregates more readily (1–2 d after appearance) than the more compact spheres in chemically defined medium.

**Pituitary spheres are clonal in origin and can generate secondary spheres**

Because pituitary cells have a tendency to reassociate, as evident from the eventual formation of larger aggregates in the sphere cultures as well as from our previous work using reaggregate pituitary cell cultures (44, 45), it was determined whether pituitary spheres originated from one cell. First, low-density cultures (1,000 cells/ml instead of 100,000) were scrutinized, and spheres were also obtained under these conditions (about one sphere per 4,000 cells). In view of the low incidence of sphere formation, an approach of growing spheres from single cells cloned by FACS or limiting dilution
was hardly achievable. Therefore, the following commonly applied approach to examine clonality of floating spheres was used (18). AP cells from transgenic eGFP-expressing and WT mice (both with identical FVB genetic background) were mixed in equal numbers and cultured under sphere-forming conditions. The spheres obtained were entirely composed of either fluorescent (GFP⁺) cells or nonfluorescent (WT) cells (Fig. 5, A and B). GFP⁺ and WT spheres were present in equal proportions. No mixed spheres were observed, whereas GFP⁺ and WT cells were intermingled in the clumps (Fig. 5, A and B). In some larger aggregates, clonal (GFP⁺) spheres could be discerned in the center (Fig. 5C). To support the idea that pituitary spheres developed and grew in size by cell division, BrdU was added to the cultures (on d 1). The majority of cells in d 3 spheres were labeled with BrdU (Fig. 5D), whereas in the irregular cell groups (clumps), only some cells had incorporated BrdU under the stationary culture conditions used (data not shown).

To assess their potential for self-renewal, primary pituitary spheres were dissociated into single cells, and cells were seeded under similar conditions as for primary sphere generation, except for the cell number (10,000 cells/ml instead of 100,000 cells/ml). Secondary spheres developed in time like primary spheres and were similar in morphology and size (Fig. 6). Moreover, when primary spheres were recovered from the larger aggregates, the dissociated cells of the sphere proper gave rise to secondary spheres, but not the dissociated peripheral cells of the aggregates remaining after recovery of the primary sphere. Secondary spheres were low in number (about five per 20,000 cells) and did not considerably grow in size after d 4–6 in culture. The paucity and limited growth of secondary spheres made the study of third and further generation sphere formation unfeasible.

**Pituitary spheres show an expression profile resembling that of Sca1high SP cells**

Spheres were individually picked and analyzed by RT-PCR for the expression of stem/progenitor cell-associated factors (Oct-4, nestin, prominin-1, Notch1, Hes1, Fzd receptors, and Ptc1), embryonic pituitary transcription factors (Lhx3, Lhx4, and Prop1), hormone cell markers (αGSU, GH, POMC, PRL, and LHβ), and the FS cell protein S100. Expression in spheres was compared with expression in individual irregular cell groups (clumps) of comparable size, picked from the same cultures. Results are shown in Fig. 7.

Oct-4 mRNA expression was detected in 80% of the sphere samples tested. All the spheres contained nestin and Hes1 mRNA, and 80% expressed Notch1 mRNA. Fzd receptor mRNA was amplified using the consensus PCR primers from 80% of the spheres, Ptc1 mRNA from 75%, and prominin-1 mRNA from 60% of the spheres. In contrast, the expression of all these factors and signaling molecules was not detected or was much less frequently detected in the clumps. The expression of Lhx3 and Lhx4 was detected in half the spheres, either alone or together. Of the five spheres analyzed for both Lhx3 and Lhx4, one contained only Lhx4 mRNA, one contained both Lhx4 and Lhx3 mRNA, and three contained only Lhx3 mRNA. Lhx3 was also detected in the clumps (76%), in contrast to Lhx4. Prop1 mRNA was not found in the spheres or in the clumps.

The majority (70%) of the spheres did not express any hormone (Fig. 7). In 20% of the spheres, αGSU mRNA was
detected, alone or in combination with POMC mRNA. None of the pituitary spheres tested contained mRNA of PRL or LH at a detectable level. In contrast, PRL and/or LH mRNA was detected in 89% and 33% of the clumps, respectively. GSU and/or GH mRNA were amplified from 80% of the clumps, and POMC mRNA was amplified from 100%. S100 mRNA was observed in some clumps (7%), but not in spheres.

Similar differences in mRNA expression pattern between spheres and clumps were found when total RNA from an identical number of sphere cells or clump cells was analyzed by semiquantitative RT-PCR as performed for a selection of the markers (see Materials and Methods; data not shown). These control experiments indicate that the single sphere or clump lysis method used above (see Materials and Methods) did not affect RT-PCR outcome, and that unintentional differences in cell number (which we tried to avoid by picking spheres and clumps of similar sizes) were not responsible for the specific expression profiles obtained.

The expression of nestin and Lhx3 was also detected at the protein level in the spheres (in almost all and in 70% of the spheres, respectively; Fig. 5E and data not shown). In accordance with the mRNA expression pattern, nestin and Lhx3 protein were also detected in some or a large part of the clumps, respectively. The expression of αGSU, POMC, and other hormones (combined analysis for PRL, GH, and TSHβ) was detected in cell clumps, but not in spheres (Fig. 5, F and G, and data not shown).

**Sphere-initiating cells of AP segregate to SP**

In view of the largely overlapping expression profile of SP and sphere cells, we examined whether there was a relationship between these cells and, in particular, whether the sphere-initiating cells of the AP displayed an SP phenotype and purified within the SP. GFP SP cells as well as GFP MP cells were isolated by FACS from APs of eGFP transgenic mice and cocultured under sphere-growing conditions with unsorted AP cells from WT mice at a ratio of 10,000/200,000/dish. In the GFP SP cocultures, spheres (10–20/dish) were detected that were homogeneously GFP (Fig. 5H) in addition to the normal number of spheres (n ≤ 30–50) consisting of WT cells only. In the GFP MP cocultures, no GFP spheres were found, but only spheres consisting of WT cells (Fig. 5I). GFP cells were present in clumps and aggregates of the GFP MP cocultures (Fig. 5I), indicating that longer retention of Hoechst dye was not toxic to the MP cells. Also, the viability of SP and MP cells was identical after sorting (~90%), as checked by the trypan blue exclusion test.

**Discussion**

In the study presented here, we show that the adult mouse AP contains a population of cells with verapamil-sensitive dye efflux capacity, a characteristic originally assigned to HSC in bone marrow (6) and subsequently to stem/progenitor cells in a number of other tissues, such as forebrain,
mammary gland, skeletal muscle, and testis (7, 8, 10, 11). We found that this so-called SP is enriched in cells that express stem/progenitor cell-connected factors, such as Sca1, Oct-4, Nanog, nestin, prominin-1, and Bmi-1, and members of the Notch (Notch1 and Hes1), Wnt (Fzd8), and Shh (Ptch1) signaling pathways functional in stem cell homeostasis as well as in pituitary embryogenesis. Moreover, the SP contains cells expressing the early embryonic pituitary transcription factor Lhx4, whereas it is depleted from cells expressing Lhx3 and from differentiated cells (expressing hormones). Finally, the SP includes cells that can clonally expand to spherical structures, which in several other tissues are known to grow from the resident stem/progenitor cells (12–19). These pituitary spheres display an expression profile largely overlapping with that of the Sca1<sup>high</sup> SP cells. Thus, our study describes a hitherto unobserved cell population in the adult AP with characteristics suggestive of an early embryonic and stem/progenitor cell phenotype.

The AP Sca1<sup>high</sup> SP, characterized in the present study, does not appear to be contaminated with HSC, because neither CD45<sup>+</sup> nor c-Kit<sup>+</sup> and CD34<sup>+</sup> cells were detected within this fraction. In fact, this was not expected, because mice were perfused before harvesting the pituitaries, removing potential HSC present in the intrapituitary blood. Furthermore, pituitary Sca1<sup>high</sup> SP cells were not able to rescue mice from lethal irradiation (Vankelecom, H., unpublished observations).

The transcription factors Oct-4 and Nanog, to date only reported in the archetype stem cell (ES cell) (22, 23) and in some candidate adult stem/progenitor cells obtained in culture (24), are expressed in the adult pituitary SP. To our knowledge, this is the first report of Oct-4 and Nanog expression in an adult organ <i>ex vivo</i>. Oct-4 and Nanog play an essential role in keeping ES cells undifferentiated and self-renewing (22, 23). Along the same line, the protooncogene product, Bmi-1, and the membrane antigen, Sca1, have been demonstrated to be functional in HSC and NSC self-renewal (26, 27, 48). It is tempting to assign similar roles to these factors within the context of the adult AP. The function of the intermediate filament protein nestin and of the membrane glycoprotein prominin-1 in stem/progenitor cell biology is as yet not clear.

The adult pituitary SP displays a striking embryonic expression profile. Lhx4 is exclusively found in SP, whereas the related Lhx3 is only detected within MP cells. Lhx3 and Lhx4 play cardinal roles in formation and fate commitment of Rathke’s pouch as well as in AP cell lineage specification, expansion, and terminal differentiation (41, 42). A recent study placed Lhx4 upstream of Lhx3 during pituitary development by showing that Lhx4 is required for the timely activation of Lhx3 (51). The same study also identified Lhx4 as a critical factor for survival and maintenance of the putative progenitor cells in the embryonic pituitary. Lhx3 remains well expressed in the adult gland (41, 42). In contrast, Lhx4 expression shows a marked diminution from E15.5 and is only detected at a very low level in the adult AP (42). Our finding of selective Lhx4 expression in the SP indicates that Lhx4-expressing cells of the adult pituitary segregate to the SP. These SP cells may represent enduring early embryonic cells maintained by Lhx4 expression, which is in line with the above-mentioned role for Lhx4 in embryonic progenitor cell maintenance (51), or alternatively, cells in the adult pituitary capable of using the embryonic program. In both cases, these cells can further differentiate and thus function as source cells for new endocrine cells within the adult gland. Lhx3 has been suggested to remain expressed in the differentiated hormone cells considering its widespread presence in the adult AP (41, 42). Along the same line, Lhx3 has been shown to regulate the expression of Pit-1, αGSU, FSHβ, and, in synergy with Pit-1, GH and PRL (41, 52). Our finding of Lhx3 expression specifically in the MP is in agreement with Lhx3 activity in further differentiated pituitary cells. Prop1 was not detected in the SP, but only in the MP. Prop1 is essential for development of Pit-1-dependent lineages (49), but has recently also been suggested to play a role in the migration of putative AP progenitors from the periplumeral region of Rathke’s pouch to the developing AP while they differentiate (53). Thus, SP cells in the adult pituitary do not seem to express this property.

Other protagonists of pituitary early embryonic development, similar to Lhx4, are detected at higher levels in SP than in MP, viz. members of the Notch, Wnt, and Shh pathway, which are, at the same time, key regulators of the balance between self-renewal and fate determination in stem cells (28–36). A first striking observation is the markedly higher expression of Notch1 and one of its downstream effectors (Hes1) by SP cells. The expression of both members suggests activated Notch signaling in pituitary SP cells or in at least some of them. Notch signaling has only very recently been implicated in pituitary embryogenesis based on the detection of Notch pathway components in a temporally and spatially restricted manner (40). Interestingly, Hes1 expression is restricted to the proliferating cells adjacent to the residual lumen of Rathke’s pouch, which are generally regarded as embryonic progenitor cells (40, 53, 54), whereas it is excluded from the differentiating cells in the developing AP. In the central nervous system, Hes1 is a key component in preserving the self-renewal capacity of NSC and repressing their commitment to a neuronal fate (55). Our finding of Hes1 in the adult pituitary SP could therefore be consistent with a role in maintaining these cells, or at least some of them, in an undifferentiated phenotype and capable of self-renewing.

Another signaling system recently implicated in pituitary organogenesis is the canonical Wnt pathway (37, 56, 57). Our finding of differential expression of Fzd receptors in SP and MP, i.e. more Fzd8 in SP and more Fzd2 in MP, suggests a divergent function for Wnt signals in each of these two cell populations within the adult AP. Previously, the expression of Fzd2 (= Fzd10) was detected not only in the early embryonic pituitary, but also in the adult gland (56) as well as in pituitary cell lines (G7, GHFT1, αTSH, and αT3) that represent different stages of committed or differentiated hormone cell lineages (56). Our finding of higher Fzd2 expression in MP is consistent with Fzd2 being present in committed or differentiated cells. Fzd8, on the other hand, has (together with Fzd4, Fzd6, and Fzd7) mainly been associated with a stem cell phenotype (9), and our finding of Fzd8 expression predominantly in SP seems to be in line with this.

A final signaling pathway analyzed in the present study and implicated in pituitary embryonic development is that mediated by Shh. Shh is initially expressed uniformly
throughout the oral ectoderm, but its expression is excluded from the nascent Rathke’s pouch on E9.0–9.5 (37, 39), creating a molecular boundary within the continuous ectoderm. Its receptor, Ptch1 (which silences signaling of Smoothened in the absence of Shh), remains expressed in the invaginating pouch, indicating that cells of the nascent pituitary are receiving a Shh signal, because Ptch1 is a direct transcriptional target of Shh. Using genetically modified mice, it was confirmed that Shh signaling from the expression boundary within the oral epithelium is required for proliferation, cell type determination, and, in cooperation with FGF8, proper expression of Lhx3 during pituitary organogenesis (39). We measured higher mRNA levels of Ptch1 receptor in SP than in MP. The Ptch1-expressing SP cells in the adult pituitary may thus also represent cells of embryonic phenotype that receive Shh signals.

Taken together, identification in the adult pituitary of signaling systems that are functional during embryonic development of the gland is in line with the growing idea that embryonic morphogens and signals persist in adult tissues to maintain life-long cellular replacement, and that cells with preserved embryonic characteristics may function as stem/progenitor cells in the adult tissue (19, 31, 34). In view of this concept, it is tempting to hypothesize that SP cells, at least some of them, represent stem/progenitor cells in the adult pituitary maintained or triggered by (local) embryonic signals to recapitulate development. To substantiate this hypothesis, it will be of great interest to define how the expression of the particular factors identified is distributed over individual SP cells. Alternative hypotheses, however, should not be overlooked. SP cells, or at least some of them, may correspond to cells that have dedifferentiated and are capable of generating new hormone-producing cells using a program mimicking the embryonic process (58). Dedifferentiated cells may represent a flexible pool of reserve cells from which specific cell types can develop according to the endocrine needs of the organism. On the other hand, SP cells may embody intermediates in transdifferentiation events that may occur via an immature (uncommitted) state (58). Such phenotypic switches between different cell types are not uncommon in the pituitary (59–61).

The AP SP also contains FS cells, because S100 mRNA is detected in the SP, and some SP cells (~6%) have the capacity to take up the dipeptide β-Ala-Lys-Ne-AMCA, a characteristic property of FS cells in the AP (46). Because FS cells comprise approximately 7% of the mouse AP cells (50), and the SP comprises only 1.7%, it is clear that only a small subpopulation of FS cells purifies with the SP. Some investigators have in the past suggested a stem cell role for FS cells (46), but convincing evidence is still lacking. The remaining S100 mRNA-expressing cells are found in the cell population outside the SP and MP sorting gates in the FACS plot (the non-SP+/non-MP).

In a complementary approach toward stem/progenitor cell identification, we were able to obtain clonal spheres in cell cultures from mouse AP. Sphere cultures have been instrumental in the identification and characterization of stem/progenitor cells from a number of tissues (12–19). Interestingly, sphere-initiating cells display an SP phenotype and segregate to this Hoechstlow population, as has also been reported for neurosphere-forming cells (9, 10). Moreover, pituitary spheres exhibit an expression profile largely in common with that of ScAlhigh SP cells: a high percentage of the pituitary spheres express factors known to be present or functional in stem/progenitor cells and embryonic pituitary cells, whereas only a limited number express markers of mature pituitary cells (hormones). Again, the expression of both Notch1 and Hes1 is very striking and suggests that the Notch signaling pathway is activated in spheres. There are also some differences, however, between SP and the sphere expression profile. S100 mRNA is not found in spheres, whereas FS cells are present in the SP, although at low number. The opposite is true for Lhx3, which is detected in spheres, but not in SP. The expression of Lhx3 in pituitary spheres may be associated with the generation of further differentiated cells within the sphere. As demonstrated in neurospheres and mammospheres (clonal spheres from the mammary gland) (10, 16, 35), it is not inconceivable that the pituitary sphere is composed of one or only a limited number of sphere-initiating, self-renewing cells and, for the remainder, of further differentiated, more restricted cells with no self-renewal, but proliferative capacity. During progression along the differentiation path, Lhx4 expression may decrease as occurring during embryonic development, potentially explaining why Lhx4 is not detectable anymore in some spheres. Along the same line, the expression in some spheres of aGSU and POMC, the two earliest markers of embryonic AP cell differentiation (43, 63), may be a first sign of (early) differentiation within the sphere. Again, it will be of great interest to identify the expression at the individual cell level in the sphere to substantiate this hypothesis.

About 0.02% of the AP cells possess sphere-forming capacity, comparable to the proportion of sphere-initiating cells in pancreas (18) and skin (19), but smaller in number than their counterparts in mammary gland (16). In the pituitary spheres obtained, cell proliferation appears to cease at a certain time point in culture. A similar halt in sphere growth has been reported for pancreatic spheres (18). The reason is unclear at present, but may relate to the lack of essential cell-cell contacts with other pituitary cells (supportive niche cells?) and/or of crucial juxtacrine growth factors from these cells. One argument in favor of this hypothesis is that when other AP cells are attached to the spheres, as in the larger aggregates, the spheres recovered are bigger and contain more cells. The number of cells in pituitary spheres is comparable to the number reported in skin-derived spheres and mammospheres (16, 19, 35), but lower than that in pancreatic spheres and neurospheres (10, 18). Only a few secondary spheres were generated from primary sphere cells. The generation of limited numbers of (small) secondary spheres has also been reported for pancreatic spheres (18). The reasons for this may be similar to those for limited primary sphere expansion.

In conclusion, we show in this study that the mouse AP contains a population of cells with SP characteristics, as found in other tissues. This population is enriched in cells that express markers and signaling molecules indicative of stem/progenitor cells and contains cells that generate clonal spheres with comparable expression profiles. Furthermore, at least some of these cells express members of signaling
systems (Notch, Wnt, and Shh) and transcription factors (Lhx4) that are essential during pituitary embryogenesis, suggesting that they represent cells with preserved embryonic nature that may function as source cells for new hormone-producing cells in the adult gland. Additional elaborates should provide insight into the potential of these cells, or at least some of them, to differentiate into multiple lineages of the AP to lend further support to such a stem/progenitor cell phenotype.

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References

17. Liu BY, McDermott SP, Khwaja SS, Alexander CM 2004 The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci USA 101:4158–4163
22. Ratsch LM, Ross SA, Cook S, Dunwoodie SL, Camper SA, Thomas PQ

Downloaded from https://academic.oup.com/endo/article-abstract/146/9/3985/3998 by guest on 16 December 2017
46. Lewis R 2004 Dedifferentiation: more than reversing fate. Pinning down the origins of a slippery cell state. Scientist 18:20

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