Sca1⁺ murine pituitary adenoma cells show tumor-growth advantage

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Abstract

The role of tumor stem cells in benign tumors such as pituitary adenomas remains unclear. In this study, we investigated whether the cells within pituitary adenomas that spontaneously develop in *Rb*^{+/–} mice are hierarchically distributed with a subset being responsible for tumor growth. Cells derived directly from such tumors grew as spheres in serum-free culture medium supplemented with epidermal growth factor and basic fibroblast growth factor. Some cells within growing pituitary tumor spheres (PTS) expressed common stem cell markers (Sca1, Sox2, Nestin, and CD133), but were devoid of hormone-positive differentiated cells. Under subsequent differentiating conditions (matrigel-coated growth surface), PTS expressed all six pituitary hormones. We next searched for specific markers of the stem cell population and isolated a Sca1⁺ cell population that showed increased sphere formation potential, lower mRNA hormone expression, higher expression of stem cell markers (Notch1, Sox2, and Nestin), and increased proliferation rates. When transplanted into non-obese diabetic-severe combined immunodeficiency gamma mice brains, Sca1⁺ pituitary tumor cells exhibited higher rates of tumor formation (brain tumors observed in 11/11 (100%) vs 7/12 (54%) of mice transplanted with Sca1⁺ and Sca1[–] cells respectively). Magnetic resonance imaging and histological analysis of brain tumors showed that tumors derived from Sca1⁺ pituitary tumor cells were also larger and plurihormonal. Our findings show that Sca1⁺ cells derived from benign pituitary tumors exhibit an undifferentiated expression profile and tumor-proliferative advantages, and we propose that they could represent putative pituitary tumor stem/progenitor cells.

Key Words

- ▶ pituitary
- ▶ neoplasia
- ▶ pathogenesis

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Introduction

Pituitary tumors are invariably benign neoplasms identified in ~25% of unselected autopsy specimens, and may cause considerable morbidity due to excess hormone secretion and/or compression of surrounding brain structures (Melmed 2003). However, precisely how pituitary adenomas develop and progress and the molecular bases of their unique features are poorly understood.

Stem cells are unspecialized cells with the ability to self-renew (ability to go through numerous cycles of cell division while maintaining the undifferentiated state) and differentiate into specialized cell types. Broad types of stem cells include embryonic and non-embryonic (or adult) stem cells. Adult stem cells that act as a repair system not only replenish specialized cells but also maintain normal

regenerative organ turnover (Preynat-Seauve & Krause 2011). The cancer stem cell theory posits that neoplasms, like physiologic tissues, contain a small population of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These tumor stem cells have the capacity to both divide and expand the cancer stem cell pool and to differentiate into heterogeneous non-tumorigenic cancer cell types that usually appear to constitute the bulk of cancer cells within the tumor (Clarke et al. 2006, Frank et al. 2010). Tumor stem cells have been described in hematologic and solid cancers, and their existence may explain tumor recurrence observed after therapies (Visvader 2011, Magee et al. 2012). According to the cancer stem cell hypothesis, failure to eliminate cancer stem cells due to anti-cancer therapy resistance results in repopulation of tumor bulk after initial apparent remission (Clarke et al. 2006, Frank et al. 2010).

However, the question of what potential role tumor progenitor/stem cells play in the progression of benign tumors in general, and pituitary adenomas in particular, remains unclear. In this study, we report that the small subpopulation of Sca1-expressing pituitary tumor cells possesses progenitor cell characteristics and tumor-growth advantage.

Materials and methods

Animals

Experiments were approved by Institutional Animal Care and Use Committee. Pituitary tumors were obtained from *Rb*+/- mice that spontaneously develop pituitary tumors with high penetrance (Jacks et al. 1992, Leung et al. 2004). *Rb*+/- mice of a 129/Sv genetic background were purchased from Jackson Laboratory (Sacramento, CA, USA) and backcrossed for at least five generations to a C57BL/6 parental genotype. Animals were genotyped by PCR as previously described (Donangelo et al. 2006). Mice were killed when cranial bump corresponding to pituitary tumor was visible, or when they became unthrifty.

Immunocompromised, non-obese diabetic-severe combined immunodeficiency (NOD scid) gamma (null) (NSG) female mice were purchased from Jackson Laboratory, and used for transplantation of pituitary tumor cells into the brain. Twenty-four hour urine collection for measurement of corticosterone was carried out in metabolic cages, 12 weeks after surgery. Mice were allowed to acclimate for more than 24 h before urine collection. At the time of killing of NSG mice, terminal general anesthesia was administered using i.p. ketamine and

dexmedetomidine injection, and the animals were perfused with PBS, followed by 4% paraformaldehyde. Brain was fixed in 4% paraformaldehyde and embedded in paraffin. Serum was stored at -80 °C until hormone assays were performed. Adrenal glands were removed from the adjacent tissues, weighed, fixed, and processed for hematoxylin and eosin (H&E) staining.

Isolation and culture of pituitary tumor spheres

Pituitary tumors were dissociated into single cells with Neural Tissue Dissociation Kit (P) (Miltenyl Biotec., San Diego, CA, USA), the cells were counted and plated into ultra-low attachment surface culture dishes (Corning, Inc.) at the density of 100 000 cells/ml in Neurocult proliferation kit medium (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF), 0.0002% heparin (Sigma-Aldrich), and antibiotic-antimycotic 1% (Life Technologies). For simplicity, this will be referred to as sphere medium. After 8–12 days of culture, pituitary tumor spheres (PTS) were mechanically dissociated by pipetting up and down using fire-polished Pasteur pipette and replated in a fresh sphere medium. Each time this process was performed, it was counted as a passage.

In vitro differentiation assay

The PTS from passages 7–9 were centrifuged at a short low speed (3 min, 80 *g*) for separation of spheres from the isolated cells and most of supernatant is removed. The cell culture at this later stage consists PTS and virtually no cell aggregates were observed. Spheres were resuspended in the sphere medium without growth factors and transferred to matrigel-coated chambered slide Lab TeK II (Nunc, Rochester, NY, USA) for 9 days and the medium replenished every 3 days. Matrigel was diluted in a ratio of 1:10 in the medium at 4 °C, incubated at 37 °C for 1 h, followed by removal of excess unbound matrigel. The spheres in matrigel were then fixed in 4% paraformaldehyde, and immunofluorescence was performed as described below. For differentiation assay with serum, 5% FBS was added to the medium.

Isolation of Sca1-positive population from pituitary tumors

Sca1⁺ and Sca1⁻ cells were obtained from pituitary tumor single-cell suspension by fluorescent-activated cell sorting (FACS) in Dako MoFlo Cell Sorter (Carpinteria, CA, USA) after incubation using Anti-mouse Ly-6A/E (Sca1)-FITC

(eBioscience) in 1% BSA (Sigma) in DMEM/F12 medium (Life Sciences). Anti-mouse CD45-PE (eBioscience) was also added for *in vitro* experiments to exclude CD45⁺ cells, and 7-amino-actinomycin D was used to identify and exclude the dead cells. For clonogenic assay, dissociated PTS, or Sca1⁺ and Sca1[−] pituitary tumor cells were singly plated or plated at one cell/20 mm² density in the sphere medium. For *in vivo* experiments, one to three tumors were used to obtain sufficient Sca1⁺ cells for brain cell transplantation.

PTS and Sca1⁺ and Sca1[−] pituitary tumor cell proliferation assays

To evaluate whether PTS grow as a result of cell division, 10 μmol 5-bromo-2'-deoxy-uridine (BrdU) was added to the sphere medium of dissociated cells derived from PTS. After 3 days, all spheres and other cells were transferred to poly-D-lysine (Sigma-Aldrich)-coated chambered slide for overnight attachment. The cells were then fixed with 4% paraformaldehyde and analyzed with BrdU Labeling and Detection Kit II (Roche). Ki67 staining was also performed to assess the proliferation of PTS (see 'Immunostaining' section).

For water-soluble tetrazolium salt (WST)-proliferation assay, FACS-sorted Sca1⁺ and Sca1[−] pituitary tumor cells were plated at the density of 2×10^3 cells/well in 96-well plates with the sphere medium supplemented with 5% FBS. WST-1 reagent (Roche Molecular Biochemicals) was added (1:10) at the indicated times and incubated for 8 h at 37 °C in a humidified atmosphere maintained at 5% CO₂, after which absorbance was measured at 450 nm.

In vivo tumor formation assay

NSG 8–10 weeks old female mice were anesthetized with ketamine (75 mg/kg) and dexmedetomidine (0.5 mg/kg) and placed on Leica Angle Two animal stereotaxic instrument. A burr hole was created in the skull with a dental drill and an injection needle was introduced stereotaxically 1 mm posterior and 2.5 mm lateral to bregma, at a depth of 3.0 mm (right putamen–striatum). Each mouse received one injection containing 10⁵ cells diluted in 2–2.5 mcl of hibernation medium (30 mM KCl, 5 mM glucose, 0.24 mM MgCl 2×6H₂O, 10.95 mM NaH₂PO₄×H₂O, 5 mM Na₂HPO₄×2H₂O, pH 7.2). Atipamezole (1 mg/kg) was administered after surgery to reverse the effects of general anesthesia. Eleven mice received Sca1⁺ pituitary tumor cell transplants, and 12 mice received Sca1[−] cell transplants, and procedures were matched so that mice received both cell types on the

same day or 1 day apart. Mice were monitored closely and killed between 12 and 21 weeks after transplantation when signs of illness developed, in which case mice having received the matched cell counterpart were also killed, or at 15 weeks for the 14 mice that had tumor growth monitored by a series of magnetic resonance imaging (MRI).

Brain MRI

MRI experiments formice brain were performed serially in 14 mice (six received Sca1⁺ and eight Sca1[−] pituitary tumor cells) on Bruker BioSpec 94/20. The animals were anesthetized with 1.8% isoflurane in 100% oxygen and placed in a body holder which was inserted into the MRI coil. Respiratory rate was monitored using a vital sign monitor (SA Instruments, Stony Brook, NY, USA). Specifications for image acquisition were as follows: i) hardware: magnetic field strength 9.4T, field BGA12-S (Bruker, Billerica, MA, USA) gradient, 660 mT/m and 4570 T/m per s slew rate transmit coil, 72 mm diameter circular polarized coil (T10325V3, Bruker) receiver coil, 4 channel mouse brain array coil (T11071V3, Bruker); ii) acquisition protocol and parameters: method, spin-echo; field of view, 1.80×1.80 cm; acquisition matrix, 196×196; slice thickness, 0.50 mm; number of slices, 30; in-plane resolution, 92×92 μm; repetition time, 900 ms; echo time, 8 ms; averages, 4; and total time, 11 min 45 s. No acceleration or zero filling was applied during reconstruction of the data from the four acquisition channels.

Tumor size measurement

To calculate tumor size, paraffin-embedded brains were sectioned at four-microns thickness in the region of pituitary tumor cell transplantation (striatum), and three every 15 sections were stained with H&E. By this method, tumors as small as about 60 microns in length could be identified. All stained slides were scanned with PathScan Enabler IV Histology Slide Scanner (Meyer Instruments, Houston, TX, USA) 7200 dpi, and the tumor area (pixels) was calculated with Image J (Abramoff et al. 2004) by manually drawing a line around the edge of the tumor on each section. The largest area of each tumor was used for comparison of tumor area size.

Brain tumors derived from pituitary tumor cell transplants were identified on MRI sections as enhanced mass. Tumor volume (mm³) calculation was performed by manually drawing a line along the edge of the tumor in a magnified image of every section in which tumor was visible, then by summing the tumor area (mm²) of all sections and multiplying result by section thickness (0.5 mm).

Immunostaining

PTS were prepared for cryosection by fixing in 4% paraformaldehyde, followed by PBS wash and 30% sucrose in 0.1 M phosphate buffer incubation for 24 h. Spheres were then washed with PBS, pellet resuspended in 10% gelatin, embedded in Tissue Tek OCT compound (Sakura, Alphen aa den Rijn, Netherlands), and cryosectioned at ten micron thickness for immunostaining. For Ki67 and Sca1 PTS immunofluorescence and for Sox2, Nestin, and GFAP staining of Sca1⁺ and Sca1⁻ pituitary tumor cells; the cells were incubated overnight in ECL-cell attachment matrix (Millipore, Billerica, MA, USA)-coated chambered slides and fixed with 4% paraformaldehyde.

Immunostaining was performed as described previously (Donangelo et al. 2006). Antibodies against the following proteins were used at indicated dilutions: ACTH (rabbit 1:200–1:1000), LH (guinea-pig 1:200–1:500), TSH (rabbit 1:200–1:500), PRL (guinea-pig 1:200), GH (rabbit 1:200), α GSU (guinea-pig 1:200–1:500) (National Hormone and Peptide Program, NIDDK and Dr Parlow, Harbor–UCLA Medical Center, Los Angeles, CA, USA), prolactin (goat 1:200–1:500, Santa Cruz, Dallas, TX, USA), α -MSH (rabbit 1:200, Phoenix Pharm, Providence, UT, USA), Sox2 (Rabbit 1:500, Millipore), Nestin (mouse monoclonal 1:200, Abcam, Cambridge, MA, USA), GFAP (mouse monoclonal 1:100, Millipore or goat 1:200, Abcam), S100 β (rabbit 1:200, Abcam), Ki67 (rabbit 1:200, Abcam), and Sca1 (rat 1:50–1:1000, Abcam). In paraffin-embedded tissue samples, antigen retrieval with 10 mM citrate buffer was performed (at 98 °C for 45 min and cooling for 20 min). Sca1 immunodetection in tissue samples was performed with Rat-on-mouse HRP Polymer (Biocare, Concord, CA, USA), followed by DAB (Dako) and Mayer's hematoxylin counterstaining. For immunofluorescence of spheres on matrigel surface, 0.05% Triton-X was added to the primary antibody solution to allow its penetration into the center of the sphere. DAPI (Molecular Probes) was used for counterstaining of nuclei. Samples were mounted in ProLong Gold antifade reagent (Life Technologies).

Confocal microscope images were obtained using a TCS-SP 5 $\times$ confocal scanner (Leica Microsystems). We used sequential laser excitation at 405, 488, 568 nm, and emission ranges from 420 to 480 nm for DAPI, 495 to 525 nm for Alexa 488, and 590 to 620 nm for Alexa 568. For imaging of PTS sections, confocal images were captured in a Z-series with slice distance between 0.7 and 1 μ m. Each fluorescent label was imaged separately in a sequential acquisition mode.

Real time RT-PCR

Total RNA was extracted with RNeasy Micro Kit (Qiagen) and was reversed transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative PCRs and analysis were carried out using iQ5 Multicolor Real-Time PCR Detection Systems (Bio-Rad Laboratories) as described previously (Fukuoka et al. 2011). TaqMan gene expression assays for mouse *Pomc*, *Gh*, *Prlr*, *Lhx3*, *Ly6a* (*sca*-1), *Cga*, *Actb*, *B2m*, and *18s* (*Rn18s*) were purchased from Applied Biosystems. For *Notch1*, certified RT² qPCR Primer Assay and *Actb*, *B2m*, and *18s* primers were purchased from SuperArray, and qPCR amplification was carried out with SYBR Green PCR Master Mix (Applied Biosystems).

Hormone assay

Mouse urine corticosterone levels were measured by RIA Kit (MP Biomedicals, LLC). Serum α -MSH was measured using the Alpha-MSH Elisa Kit (DRG, Marburg, Germany). Serum IGF1 was measured by mouse/rat IGF1 Elisa Kit (ALPCO, Salem, NH, USA). All assays were performed according to the instructions provided by the manufacturer.

Statistical analysis

One-way ANOVA test was used to compare rate of sphere-forming cells, two-way ANOVA for WST assay proliferation rate, and Student's *t*-test was used for the analysis of hormones and adrenal gland weight. Analysis of relative expression of genes by qPCR was performed with the sign test. Mann–Whitney *U* test was used for comparison of brain tumor area and volume of histological and MRI samples and to analyze distribution of number of hormones expressed in brain tumor samples derived for Sca1⁺ and Sca1⁻ pituitary tumor cell transplants. Wilcoxon's signed-rank test was used for the analysis of change in tumor volume in a series of MRIs performed. All statistical tests were two-sided, and significance was defined as *P* < 0.05.

Results

Tumor sphere generation from the pituitary tumors from *Rb* +/– mice

We first attempted to derive tumor progenitor/stem cells from the pituitary tumors excised from mice with heterozygous inactivation of retinoblastoma susceptibility gene (Jacks et al. 1992). Tumor spheres were obtained by culturing enzymatically dissociated single-cell suspension plated at 100 000 cells/ml in a serum-free medium supplemented

with EGF and bFGF (sphere medium). This culturing method has previously been shown to support the growth of free-floating tumor and neural stem cells exploiting their ability to grow as spheres in nonadherent conditions, while most primary differentiated cells do not survive serum-free culture conditions (Ponti *et al.* 2005, Rietze & Reynolds 2006, Beier *et al.* 2007). The spheres were observed starting at 3 days of culture, grew in size (100–120 μm) until 10–12 days (Fig. 1a, top panel), and after 2 weeks some tumor spheres started showing dark centers likely reflecting cell death. PTS were enzymatically digested after 8–12 days and were replated as single-cell suspensions that grew into new spheres. Dissociation of tumor spheres and serial replating led to a declining number of total viable cells. There was no difference in velocity of growth and sphere size as long as sufficient numbers and concentrations of cells (100 000 cells/ml) were maintained (Fig. 1a, bottom panel). Tumor sphere cultures could be maintained for up to 12 passages, indicating that the PTS spheres exhibit limited self-renewal capacity.

We next asked whether PTS grew as a result of cell proliferation, rather than cell aggregation. Cell proliferation was investigated both by determining incorporation of the thymidine analog, BrdU, added to the culture medium for 4 days, and by analyzing the expression of Ki67 nuclear staining of the PTS after 5 days in culture. We observed that BrdU was incorporated in the replication of PTS cells (Fig. 1b, i), but not in the surrounding tumor cell aggregates (Fig. 1b, ii). Similarly, only PTS (Fig. 1b, iii arrowhead and iv), but not cell aggregates (Fig. 1b, iii arrow), exhibited positive Ki67 nuclear staining.

Sphere generation has previously been shown to correlate with stem cell number (Reynolds & Weiss 1996, Galli *et al.* 2004, Reynolds & Rietze 2005). To investigate the presence of tumor sphere-generating cells, single living cells were plated at a density of 100 000 cells/ml, and the spheres counted after 10 days. Whereas in the primary tumor cell population, the ratio of spheres per plates cells was 1.03 ± 0.28 spheres/1000 cells (mean $\pm$ S.E.M.) or 1/970 plated cells; in the disaggregated spheres in culture (secondary or tertiary), the number of spheres formed per plated cells was significantly increased (3.34 ± 0.67 spheres/1000 cells or 1/299 plated cells, $P=0.005$; and 4.2 ± 0.87 spheres/1000 cells or 1/238, $P=0.003$ for secondary and tertiary cultures respectively (mean $\pm$ S.E.M.)) (Fig. 1c).

PTS characterization

To establish the types of cells that compose spheres, we next assessed expression of stem cell markers and pituitary

Figure 1

Tumor sphere generation from *Rb* $\pm$ mice pituitary tumors. (a) Representative micrographs showing the morphology and size of tumor cells and spheres over time. (Top panel, a) Pituitary tumor spheres (PTS) were observed after 3 days in culture, and they grew in size until days 10–12. (Bottom panel, a) After 8–12 days in culture, disaggregated tumor spheres were replated as single cells for several generations, with no difference in velocity of growth or size of sphere. Scale bar: 100 μm . (b) PTS grow as a result of cell proliferation. Representative micrograph indicating DNA replication in cells within spheres assessed by immunofluorescence for BrdU (culture treated with thymidine analog BrdU) or for Ki67. (b, i) BrdU-positive immunolabeling in dividing cells within PTS. (b, ii) No BrdU incorporation was noted in surrounding cell aggregates. (b, iii) Positive nuclear staining for Ki67 was observed in PTS (arrowhead), but not in surrounding cell aggregates (arrow). (b, iv) PTS with positive Ki67 nuclear staining in higher magnification. Scale bar: 25 μm . (c) Evaluation of the number of single cells able to generate spheres. Cells were plated at the density of 100 000 cells/ml. The number of sphere-forming cells was higher in the disaggregated spheres in culture (secondary or tertiary) compared with the primary tumor cell population. Data expressed as mean $\pm$ S.E.M. of 16 different experiments. * $P<0.01$.

hormones within growing spheres. Some cells within PTS were found to express markers associated with pluripotency, including Sca1, Sox2, Nestin, CD133, and S100 β (Fig. 2a, b, c, d, e, f and g). Conversely, endocrine cells expressing any of the six pituitary hormones were scarce or not detected in the PTS (Fig. 2d, e and f). The expression of Sox2 within PTS cells increased with time in culture (nuclear Sox2 detected in ~15% of cells of 2-week spheres, ~45% of 6-week spheres, and ~55% of 9-week spheres). The expression of Sox2 and Nestin was not detected in five whole pituitary tumors derived from *Rb*^{+/-} mice.

In vitro differentiation of PTS

To evaluate the differentiating ability of hormone-negative pituitary tumor cells that grow as spheres, they were cultured in diluted matrigel-coated surface in a medium without EGF or bFGF (i.e. differentiating assay without serum). After 9 days in culture, expression of all six pituitary hormones could be detected in the cells derived from PTSs (Fig. 3a, b, c, d, e and f). Nestin expression often persisted in cells surrounding differentiated cells, and isolated cells expressing both Nestin and pituitary hormone were also noted (Fig. 3f). The expression of Sox2 and Sca1 disappeared in the PTS under differentiating conditions, and the expression of GFAP and S100 β was observed only in the isolated cells (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

To evaluate the effect of serum in PTS differentiation, the differentiation assay on matrigel was repeated with the addition of serum (5%; i.e. differentiating assay with serum). The presence of serum resulted in less prominent hormone expression, with ACTH, TSH, GH, and α GSU detected focally in the PTS cells; PRL was detected in isolated cells, while LH was not detected (Fig. 4). In the differentiation assay with serum, Nestin-positive cells were again present in the cells surrounding differentiated cells; however, Sox2, Sca1, GFAP, CD133, and S100 β were not observed (Fig. 4 and Supplementary Fig. 1). These results indicate that growth of PTS in an adherent matrigel surface is sufficient to promote their differentiation into pituitary hormone-positive cells.

Pituitary tumor Sca1⁺ cells exhibit progenitor cell phenotype and proliferative advantage

Sca1 was a frequently expressed stem cell surface antigen in PTS. To determine whether the potential of generating spheres resides in a Sca1⁺ cell subpopulation, we performed FACS to fractionate Sca1⁻ and Sca1⁺ cells from the primary pituitary tumor cell population. Sca1 was expressed in an average of 2.59% of pituitary tumor cells (min. 0.42% and max. 8.25%) derived from 35 individual pituitary tumors. The cells expressing CD45 (average 0.90%, min. 0.11%, and max. 2.06%) were excluded from the collected samples to eliminate contamination with intermingled hematopoietic cells from the tumor sample.

Figure 2

Expression of stem cell markers and hormones on pituitary tumor spheres (PTS). Representative micrographs obtained by confocal microscopy showing, (a, b, c, d, e and f) immunofluorescence staining of cells within PTS of CD133, Nestin, Sox2, S100 β , and Sca1. (d, e, f, g and h)

Immunofluorescence for hormones ACTH, TSH, prolactin (PRL), glycoprotein alpha-subunit (α GSU), LH, and GH showing negativity or expression in isolated cells. Nuclei were counterstained with DAPI (blue). Scale bars: 20 μ m.

Figure 3

Pituitary tumor spheres (PTS) placed in differentiating condition (matrigel-coated surface in the absence of EGF or bFGF, without serum) express hormones. Representative micrographs obtained by confocal microscopy. (a, b, c, d, e, and f) Immunofluorescence staining of cells within PTS for pituitary hormones (ACTH, glycoprotein alpha-subunit (α GSU), LH,

prolactin (PRL), GH, and TSH); (d and e) Nestin is expressed in cells surrounding differentiated cells. (f) Occasional cells co-expressed hormone and Nestin (arrowhead). Nuclei were counterstained with DAPI (blue). Scale bars: 20 μ m.

Sca1⁻ and Sca1⁺ cells were plated in sphere-generating conditions at similar density (100 000 cells/ml) and observed for 5–8 days after plating. Sphere-generating capacity was shown to positively correlate with Sca1 expression (Fig. 5a and b). The spheres obtained from Sca1⁺ cells showed similar morphology as those observed in cultured primary tumor cells. Sca1⁺ pituitary tumor cells proliferated faster than the Sca1⁻ cell population when plated in a serum-containing medium, as assessed by WST-1 assay (4.4-fold on day 3, $P < 0.001$; Fig. 5c). No spheres grew during clonogenic assay, neither from singly nor from very-low-density (one cell/20 mm²) plated-dissociated PTS or Sca1⁺ pituitary tumor cells (data not shown).

To establish whether Sca1⁺ pituitary tumor cells were less differentiated than their Sca1⁻ cell counterpart, we next assessed the expression of pituitary hormones by qRT-PCR (Fig. 5d). Sca1⁺ pituitary tumor cells showed significantly lower mRNA levels of *Pomc*, *Gh*, and *Prl*, while relative expression of *α Gsu* was not different between Sca1⁺ and Sca1⁻ cells. Conversely, the expression of the stem cell marker *Notch1* was higher in Sca1⁺ cells, while

no difference was observed in the expression of *Lhx3*, a marker of committed pituitary cells. Expression analysis of pituitary tumor cells by immunofluorescence showed that Sox2 was more frequently detected in Sca1⁺ cells (nuclear Sox2 present in 55% (290/528) of Sca1⁺ and 8% of Sca1⁻ (43/545) pituitary tumor cells). Sox2 and Nestin were colocalized in the majority of these cells (Fig. 5e). Sca1⁺ pituitary tumor cells also expressed stem cell markers Nestin and GFAP and lacked ACTH expression, in contrast to the same markers in Sca1⁻ pituitary tumor cells (Supplementary Fig. 2, see section on supplementary data given at the end of this article).

***In vivo* tumor growth of Sca1⁺ cells**

We next asked whether Sca1⁺ cells within pituitary tumors showed proliferation advantage compared with Sca1⁻ cells through *in vivo* transplant studies. While the ideal target would be the pituitary niche, this is located in a too deep site below the brain with difficult *in vivo* accessibility. We therefore chose to target the brain striatum which is more easily accessible. Identical

Figure 4

Pituitary tumor spheres (PTS) placed in differentiating conditions (matrigel-coated surface in the absence of EGF or bFGF, with serum) express hormone focally. Representative micrographs obtained by confocal microscopy. (a, b, c, d and e) Immunofluorescence staining of cells within PTS showing focal or isolated expression for pituitary hormones (TSH, ACTH, glycoprotein

alpha-subunit (α GSU), GH, and prolactin (PRL)). (c) GH expressed in cells within a PTS, while GFAP is absent. (d) Nestin is expressed in cells surrounding differentiated (ACTH-positive) cells. (e) Isolated PRL expression in a PTS, while Sox2 is absent. Nuclei were counterstained with DAPI (blue). Scale bars: 20 μ m.

numbers of Sca1⁺ or Sca1⁻ cells (10^5 cells) obtained by FACS of dissociated pituitary tumor cells were stereotactically transplanted into the striatum of 23 NSG mice. Sca1⁺ pituitary tumor cells exhibited higher rates of tumor formation than Sca1⁻ cells. The tumors were observed in the brains of all 11 mice that received Sca1⁺ cells and in 7/12 mice that received Sca1⁻ cells (Fig. 6a). Moreover, tumors originating from Sca1⁺ cells were larger than those detected in the subset of mice that received Sca1⁻ cells and developed tumors (Fig. 6b, Sca1⁺ cells ($n=11$): $117\,263 \pm 25\,919$ pixels and Sca1⁻ cells ($n=7$): $37\,340 \pm 18\,723$ pixels, mean $\pm$ S.E.M., $P=0.023$).

To track living tumors over time, some animals were monitored by MRI scanning. Mice with Sca1⁺ brain cell transplants showed increased rate of tumor formation both at 7 and 13 weeks after cell transplantation. Two of eight mice (25%) and 5/6 (83%) that received Sca1⁻ cells and Sca1⁺ cells, respectively, had brain tumors detected at 7 weeks, while 3/8 (38%) and 6/6 (100%) of mice that received Sca1⁻ and Sca1⁺ cells had tumors visible on MRI at the 13 weeks post-transplant. While the five tumors derived from Sca1⁺ cells identified at the 7-week MRI were

larger in the subsequent 13-week MRI (3.87 ± 0.99 and 24.92 ± 9.73 mm³ in 7- and 13-week MRI, respectively, mean $\pm$ S.E.M., $P=0.05$), the tumors derived from Sca1⁻ cells visible on MRI in 7-week study were not larger than in the 13-week imaging study. Representative brain MRI scan of mice that received Sca1⁺ and Sca1⁻ pituitary tumor cells is depicted in Fig. 6c, while Fig. 6d shows the average tumor volume from transplant of Sca1⁺ and Sca1⁻ pituitary tumor cells in the 7- and 13-week MRI.

Finally, we asked whether tumors arising from Sca1⁺ pituitary tumor cells expressed more pituitary hormones or stem cell markers than those derived from Sca1⁻ cells. Brain histology sections were studied by immunofluorescence, and hormone expression for ACTH, LH, PRL, TSH, GH, and α GSU was tested in the tumor samples (Fig. 7a). α -MSH was studied in a subset of tumors ($n=12$) and expression overlapped with ACTH. Two of seven (29%) and 10/11 (91%) of tumors derived from Sca1⁻ and Sca1⁺ pituitary tumor cells, respectively, expressed three or more hormones, i.e. tumor samples derived from Sca1⁺ cells were significantly shifted toward the expression of larger number of hormones ($P=0.026$) (Fig. 7b). Peripheral

Figure 5

Sphere generation by Sca1⁺ cells. Sca1⁻ and Sca1⁺ cells were isolated by fluorescent activated cell sorting (FACS) from cells derived from pituitary tumors from *Rb*^{+/-} mice. Sca1⁻ and Sca1⁺ cells, obtained from pituitary tumors, were plated at the density of 100 000 cells/ml in sphere-generating conditions. (a) The number of spheres obtained was counted after 5 days in culture, and the ability of pituitary tumor cells to form spheres correlated with expression of Sca1, as depicted in representative micrographs (original magnification, 200×). (b) Pituitary tumor cells expressing Sca1 formed significantly more spheres than Sca1⁻ cell counterpart. **P* < 0.01. (c) Pituitary tumor cells expressing Sca1⁺ exhibit increased proliferation compared with Sca1⁻ cell population. Pituitary tumor-derived Sca1⁺ and Sca1⁻ cells were plated in 96-well plated (2000/well) and WST-1 assay was performed at the indicated times. Values are mean ± s.e.m. ***P* < 0.001. This assay was repeated four times with different pituitary tumor samples with similar results. (d, Top panel) qRT-PCR results showing that Sca1⁺ pituitary tumor cells have lower mRNA expression levels of pro-opiomelanocortin

(*Pomc*), *Gh*, and prolactin (*Prl*), but not glycoprotein alpha-subunit (*αGsu*) compared with Sca1⁻ pituitary tumor cell population (Sca1⁺ fold-change ± s.e.m. relative to Sca1⁻ sample: *Pomc* (*n* = 15), 0.38 ± 0.14, *P* < 0.001; *Gh* (*n* = 12), 0.58 ± 0.21, *P* = 0.006; *Prl* (*n* = 6), 0.23 ± 0.05, *P* = 0.03; and *αGsu* (*n* = 15), 1.28 ± 0.48, *P* = 0.122); (d, Bottom panel) mRNA expression of Sca1 is higher in Sca1⁺ cell population, validating FACS cell collection; stem cell marker *Notch1* is highly expressed in Sca1⁺ pituitary tumor cell population, while pituitary cell marker *Lhx3* not changed suggesting that Sca1⁺ is less differentiated than Sca1⁻ pituitary tumor cell population (Sca1⁺ fold-change ± s.e.m. relative to Sca1⁻ sample: *Notch1* (*n* = 9), 75.0 ± 38.5, *P* = 0.004 and *Lhx3* (*n* = 10), 0.84 ± 0.20, *P* = 0.23). Results are expressed as average of Log10 Sca1⁺/Sca1⁻ mRNA relative expression ± s.e.m. **P* < 0.05 and ***P* < 0.01. (e) Sca1⁺ pituitary tumor cells exhibit high expression of Sox2 and Nestin compared with Sca1⁻ cells. Representative micrographs obtained by confocal microscopy. Nuclei were counterstained with DAPI (blue). Scale bar: 100 μm.

α-MSH, IGF1 levels, and 24 h urine corticosterone were unchanged between groups, and adrenal glands derived from the mice transplanted with Sca1⁺ cells had similar weight (2.86 ± 0.13 and 2.92 ± 0.18 mg for adrenals from mice transplanted with Sca1⁻ and Sca1⁺ cells, respectively, mean ± s.e.m., *P* = 0.79) and no histological changes suggestive of hyperplasia compared with adrenal glands of mice transplanted with Sca1⁻ pituitary tumor cells. Similarly, there was no difference in adrenal weights from mice that developed tumors, compared with mice that did not develop tumors (2.90 ± 0.14 and 2.85 ± 0.15 mg for adrenals from mice with and without tumors, respectively, mean ± s.e.m., *P* = 0.90).

The majority of tumors derived from Sca1⁺ pituitary tumor cells lost Sca1 expression after transplantation. Only two of 11 tumors derived from Sca1⁺ pituitary tumor

cell transplants, and none of the tumors derived from Sca1⁻ pituitary tumor cell transplants exhibited cells expressing Sca1 (Fig. 7c). The tumors that retained Sca1 expression were not noticeably different than tumors derived from transplant of Sca1⁺ pituitary cells with regard to number of pituitary hormones expressed (three and four hormones per tumor, respectively; average in Sca1⁺ tumors 3.5 hormones per tumor) and size (238 122 and 43 230 pixels, respectively; average tumor size in Sca1⁺ group 117 263 pixels).

Histological samples of brain tumors revealed stem cell marker expression in tumors derived from Sca1⁺ cells. There was abundant Nestin expression in tumors derived from Sca1⁺ cells, with the co-expression of Nestin and Sox2 in a subset of cells, while tumors derived from Sca1⁻ cells were negative for both Nestin and Sox2

Figure 6

Sca1⁺ pituitary tumor cells have increased tumor-forming and growth capacities than Sca1⁻ cells. (a) Tumor area (pixels) from the histology brain section in the area of cell transplantation is shown for all mice that received Sca1⁻ (red bars) and Sca1⁺ (blue bars) pituitary tumor cells. Brain tumor was observed in 7/12 and 11/11 of mice that received Sca1⁻ and Sca1⁺ cell transplant respectively. (b) Among cases in which brain tumor was observed, tumor area of histological samples derived from Sca1⁺ cells was significantly larger than those derived from Sca1⁻ pituitary tumor cells: Sca1⁻ ($n=7$), $37\,340 \pm 18\,723$ and Sca1⁺ ($n=11$), $117\,264 \pm 25\,919$ pixels, mean $\pm$ S.E.M., $**P < 0.01$. (c) Representative brain magnetic resonance

imaging (MRI) scan of mice that received pituitary tumor cell transplant in the striatum, showing that tumor (enhancing mass) derived from Sca1⁻ cells had minimal enlargement between 7 and 13 weeks post-transplant, while tumor derived from Sca1⁺ cells showed marked enlargement over the same period of time. (d) Mean tumor volume is larger in transplants derived from Sca1⁺ pituitary tumor cells ($n=6$) than those derived from Sca1⁻ cells ($n=8$), as per brain MRI. Seven weeks post Sca1⁻ and Sca1⁺ pituitary tumor cell transplants, the mean $\pm$ S.E.M. brain tumor area was 0.31 ± 0.21 and 3.23 ± 1.03 mm³, and at 13 weeks post-transplant the mean tumor area was 11.72 ± 11.07 and 23.51 ± 8.07 mm³ respectively, $*P < 0.05$.

(Fig. 7d). The tumors derived from Sca1⁺ pituitary tumor cell transplant also expressed CD133, S100 β , and GFAP (Supplementary Fig. 3, see section on supplementary data given at the end of this article).

Discussion

Although abnormalities in several pathways have been recognized in the pathogenesis of pituitary adenomas, no current general mechanism for pituitary tumor initiation is tenable. The tumor stem cell hypothesis represents an attractive unifying theory and postulates that deregulation of a small subpopulation of tumor cells determines tumor development and growth, and the wide range of mutations noted in the differentiated tumor cell

bulk may be subsequent genetic defects that accumulate as the tumor progresses.

In this study, we showed that murine pituitary tumors grow as free-floating spheres when cultured in a serum-free medium supplemented with EGF and bFGF. This culture system was initially described for enrichment of neural stem cells from adult mouse brain, in which most differentiated cells die after a few days while cells with progenitor/stem cell characteristics perpetuate and grow as spheres (Reynolds & Weiss 1992). PTS are enriched in stem cell markers, lacking hormone-expressing differentiated cells; however, expression of all six pituitary hormones is attained when PTS are plated in differentiating conditions. In addition, PTS exhibit self-renewal capacity for up to 12 passages when

Figure 7

Tumors derived from Sca1⁺ pituitary tumor cell transplants are plurihormonal. (a) Representative histology results for brain tumors derived from Sca1⁻ and Sca1⁺ pituitary tumor cell transplants, showing larger tumor area on H&E-stained histology sections and positivity for multiple pituitary hormones on immunofluorescence (IF) in the Sca1⁺ when compared with Sca1⁻ cell-derived samples. Micrographs obtained by confocal microscopy. Nuclei were counterstained with DAPI (blue). Scale bars: 300 μm. (b) Graph shows distribution of tumors derived from Sca1⁻ (red bar) and Sca1⁺ (blue bar) pituitary tumor cell transplants according to number of hormones expressed on IF (0–5). Two of seven (29%) and 10/11 (91%) of tumors derived from Sca1⁻ and Sca1⁺ pituitary tumor cells, respectively, expressed

three or more hormones. (c, i and ii) Sca1 expression detected in two of 11 brain tumors derived from Sca1⁺ pituitary tumor cell transplants; (c, iii) Sca1 expression in kidney histology sample (positive control). Scale bars: 50 μm. (d) Representative histology sample of brain tumors derived from pituitary tumor cell transplants, showing abundant Nestin expression in the tumors derived from Sca1⁺ cells (left panel), and co-expression of Nestin and Sox2 in a subset of cells (insert in left panel), while tumor derived from Sca1⁻ cells (right panel) are negative for both Nestin and Sox2. Micrographs obtained by confocal microscopy. Nuclei were counterstained with DAPI (blue). Scale bars: 50 μm.

recultured serially as single cells. Progenitors are typically descendants of stem cells, and are more constrained in their differentiation potential or capacity for self-renewal. Although limited self-renewal capacity suggests that PTS are composed of progenitor cells, progressive increase in number of senescent cells with passages has been shown to interfere with sphere culture-extended self-renewal capacity (Dey et al. 2009). Limited PTS self-renewal capacity occurred despite progressive increase in undifferentiated Sox2-expressing cells in sphere cultures. It is unclear whether activation of senescence pathways or suboptimal culturing techniques is responsible for limited cell renewal capacity. The PTS exhibit expression of Sca1-surface antigen, a cell marker initially described in murine hematopoietic stem cells (van de Rijn et al. 1989), also expressed in normal tissue stem cells (Welm et al. 2002) and solid neoplasms (Grange et al. 2008, Mulholland et al. 2009). Our results indicate that Sca1-expressing pituitary tumor cells exhibit undifferentiated expression profile, differentiation potential, and proliferative advantage,

suggesting that they could include putative tumor progenitor/stem cells.

Evidence supporting the existence of normal adult pituitary stem cells has recently been recognized (Castinetti et al. 2011). The marginal cell layer bordering the murine pituitary cleft is the presumptive stem/progenitor pituitary cell niche. The cells in this region express progenitor/stem cell markers (Sox2, Sox9, Oct4, and Nestin) and pituitary transcription factors (Prop1), as well as pituitary cell marker GFRa2 (a Ret co-receptor for Neurturin) (Fauquier et al. 2008, Gleiberman et al. 2008, Garcia-Lavandeira et al. 2009). These marginal zone hormone-negative cells form spheres in culture that differentiate into pituitary hormone-producing cells (Fauquier et al. 2008, Garcia-Lavandeira et al. 2009). Genetic lineage-tracing studies demonstrate that both embryonic and adult Sox2⁺ and Sox9⁺ cells are able to generate pituitary endocrine cells and contribute to organ homeostasis, proving that they are progenitors (Andoniadou et al. 2013, Rizzoti et al. 2013). In the human

pituitary, cells expressing stem cell markers, GFRA2, OCT-4, SOX2, and SOX9, were also detected around the so-called Rathke's pouch cysts (remnants of the Rathke's pouch cleft), a location comparable to the marginal zone in rats and mice, as the human pituitary lacks a demarcated intermediate lobe (Garcia-Lavandeira *et al.* 2009). Pituitaries derived from mice, rats, and chickens were reported to possess a 'side population' of cells identified by flow cytometry by their ability to efflux DNA-binding dye, Hoechst 33342. This side population is enriched with cells expressing stem cell markers, including Sca1. Sca1-expressing pituitary cells were noted to have a progenitor cell phenotype (Chen *et al.* 2009). Interestingly, results of previous studies on normal pituitary cells overlap with our observations on neoplastic murine pituitary cells, namely the significance of Sca1 and Sox2 markers in identifying putative stem/progenitor cells. In both normal and neoplastic pituitary cells: i) Sca1⁺ cells grow as hormone-negative spheres; ii) Sox2⁺ and Sca1⁺ cells at least partially overlap; and iii) Sox2 and Sca1 expression is no longer observed when hormone-negative spheres are exposed to differentiating conditions.

Stem cell marker and pituitary hormones do not colocalize in adult pituitaries (Krylyshkina *et al.* 2005, Fauquier *et al.* 2008, Gleberman *et al.* 2008, Garcia-Lavandeira *et al.* 2009). Similarly, we found no colocalization of stem cells markers and hormones in PTS or in tumors derived from Sca1⁺ pituitary tumor cells transplanted into mice brains. However, we observed co-expression of Nestin and ACTH in isolated cells derived from PTS under *in vitro* differentiation. It is unclear if this finding indicates incomplete *in vitro* differentiation.

Previous studies have addressed the tumor stem cell hypothesis in pituitary neoplasia. In mice, a model of expression of degradation-resistant Wnt/ β -catenin in Hex2-progenitor pituitary cells lead to the development of tumors resembling human craniopharyngiomas that arise from embryonic pituitary tissue (Gaston-Massuet *et al.* 2011). Overactivation of Wnt pathway in Sox2-expressing pituitary cells leads to the development of hormone-negative anterior lobe tumors, although the tumor mass was not derived from Sox2 cells targeted with the oncogenic β -catenin. It was proposed that the surrounding mutagenized Sox2 cells may drive tumor formation in a paracrine fashion (Andoniadou *et al.* 2013). In humans samples, pituitary adenomas (GH-positive and null-cell tumors) formed nonadherent spheres when cultured in serum-free media supplemented with EGF and bFGF (Xu *et al.* 2009). Human PTS from GH-positive tumor showed dispersed GH-positive cells

on immunofluorescence. However, early in culture, non-adherent spheres may contain aggregates of differentiated cells. The PTS derived from a GH-secreting tumor were transplanted in NSG mice brains, resulting in GH-positive grafts that could be serially transplanted. The tumors were, however, not observed in mice that received transplants from *in vitro*-differentiated PTS (Xu *et al.* 2009).

This study shows that Sca1⁺ pituitary tumor cell population exhibits higher rates of ectopic tumor formation and growth. Tumor stem cell phenotype of Sca1⁺ cells was also described in a murine model for breast cancer, where Sca1⁺ cells exhibit increased *in vitro* sphere-generating ability and *in vivo* tumor-initiating capacity (Grange *et al.* 2008). Another study showed that tumor induction by AKT activation in Sca1⁺ murine prostate cells results in tumor initiation (not observed with Sca1⁻ cells), and prostate cancer progression correlates with increased percentage of Sca1⁺ cells (Xin *et al.* 2005). In our study, ectopic tumors also developed from Sca1⁻ pituitary (albeit smaller and less frequent) and the tumor-growth advantage may not be restricted to the Sca1⁺ cell population.

A somatic mutation in adult pituitary stem cell may be the origin of pituitary neoplasia (Herman *et al.* 1990). Adult stem cells may be more prone to neoplastic transformation than mature differentiated cells, as they possess self-renewal capacity, and may persist in tissues for longer periods increasing the likelihood for accumulating mutations (Barker *et al.* 2009, Zhu *et al.* 2009). To evaluate whether Sca1-expressing cells originate pituitary tumors, an inducible model for Sca1 lineage-tracking system that leaves a permanent mark in Sca1⁺ cells and their descendants in combination with an *in vivo* pituitary tumor model would be required.

Ectopic tumors derived from Sca1⁺ pituitary tumor cells were more likely to express larger number of pituitary hormones than tumors derived from Sca1⁻ cells, and Sca1 expression was lost in the majority of these tumors. Taken together with the undifferentiated Sca1⁺ tumor cell features of lower mRNA hormone expression, co-expression of stem cell marker, and ability to form spheres in the sphere medium, these observations support the hypothesis that Sca1⁺ pituitary tumor cells are progenitors with multipotent differentiation ability. However, the possibility that Sca1⁺ pituitary tumor cells are already differentiated cells with lower mRNA hormone expression that has augmented hormone expression when ectopically transplanted cannot be entirely excluded.

Limitations of our study include the technical constraints of reduced cell numbers. To obtain sufficient cells to conduct experiments, pituitary tumors had to

be pooled, preventing us from deriving correlations of individual tumor Sca1 expression with proliferation and differentiation characteristics. However, as pituitary tumors in our study are derived from *Rb*^{+/−} mice with a similar genetic profile, minor biological variation among different tumors would be expected. Confirmation that Sca1⁺ pituitary cells are bona fide tumor stem cells requires conducting serial transplantation assays with limiting number of Sca1⁺ cells to test extended self-renewal and tumor-initiating capacities. In addition, evaluation of the effects of targeted elimination of Sca1⁺ cells on tumor maintenance and growth and whether Sca1⁺ is associated with chemo-resistance as described for tumor stem cells (Xu *et al.* 2009) needs to be assessed. Targeted therapy of cancer stem cells has shown to decrease tumor growth both *in vitro* and *in vivo* (Wang *et al.* 2010). Targeted depletion of Sca1⁺ cells from pituitary tumors would be required to confirm that these cells are required for tumor growth and progression.

In summary, our findings demonstrate that Sca1⁺ cells derived from murine pituitary tumors exhibit progenitor-cell features and tumor-growth advantage, supporting their role as putative tumor progenitor/stem cells. Elucidation of the molecular characteristics and role of these cells in pituitary tumor initiation and treatment requires further study that will lead to unraveling of incompletely understood mechanisms for pituitary tumor adenoma development and progression.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0229>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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