Pituitary tumors contain a side population with tumor stem cell-associated characteristics

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Abstract

Pituitary adenomas cause significant endocrine and mass-related morbidity. Little is known about the mechanisms that underlie pituitary tumor pathogenesis. In the present study, we searched for a side population (SP) in pituitary tumors representing cells with high efflux capacity and potentially enriched for tumor stem cells (TSCs). Human pituitary adenomas contain a SP irrespective of hormonal phenotype. This adenoma SP, as well as the purified SP (pSP) that is depleted from endothelial and immune cells, is enriched for cells that express ‘tumor stemness’ markers and signaling pathways, including epithelial–mesenchymal transition (EMT)-linked factors. Pituitary adenomas were found to contain self-renewing sphere-forming cells, considered to be a property of TSCs. These sphere-initiating cells were recovered in the pSP. Because benign pituitary adenomas do not grow *in vitro* and have failed to expand in immunodeficient mice, the pituitary tumor cell line AtT20 was further used. We identified a SP in this cell line and found it to be more tumorigenic than the non-SP ‘main population’. Of the two EMT regulatory pathways tested, the inhibition of chemokine (C-X-C motif) receptor 4 (CXCR4) signaling reduced EMT-associated cell motility *in vitro* as well as xenograft tumor growth, whereas the activation of TGFβ had no effect. The human adenoma pSP also showed upregulated expression of the pituitary stem cell marker SOX2. Pituitaries from dopamine receptor D2 knockout (*Drd2*^{−/−}) mice that bear prolactinomas contain more pSP, Sox2⁺, and colony-forming cells than WT glands. In conclusion, we detected a SP in pituitary tumors and identified TSC-associated characteristics. The present

Key Words

- pituitary
- adenoma
- side population
- epithelial–mesenchymal transition
- tumor stem cells
- cancer stem cells
- stem cells
- Sox2
- Cxcr4

study adds new elements to the unraveling of pituitary tumor pathogenesis and may lead to the identification of new therapeutic targets.

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Introduction

Pituitary tumors are generally benign adenomas. Radiology and autopsy, being performed for reasons other than pituitary-related medical ones, show a high prevalence of 10–15% in the overall population, although most of these lesions are small and asymptomatic (Daly *et al.* 2009, Melmed 2011). Clinically relevant pituitary adenomas have a lower prevalence of approximately 0.1% (Daly *et al.* 2009), but they still represent the third most frequent intracranial neoplasm, after meningiomas and gliomas. Tumorigenesis in the pituitary can pose serious medical complications. Tumors may hypersecrete one or more of the pituitary hormones, which can lead to severe endocrine disturbances and pernicious effects on the many physiological processes that are governed by the gland. Growth hormone (GH)-producing adenomas (somatotropinomas, GH-A) cause gigantism or acromegaly, adrenocorticotrophic hormone (ACTH)-producing tumors (corticotropinomas, ACTH-A) lead to Cushing's disease, and prolactin (PRL)-producing adenomas (prolactinomas, PRL-A) negatively affect gonadal status and reproduction. Conversely, a large number of pituitary tumors do not measurably secrete hormones (i.e., non-functioning adenomas (NF-A)), but their growth and expansion may lead to deficient function of the gland (hypopituitarism) and to visual disturbances as a result of the compression of the optic chiasm and nerves. In general, 30–45% of clinically diagnosed adenomas display local expansion as well as invasion of neighboring structures, such as the bony sphenoid sinus and the parasellar venous cavernous sinuses (Galland *et al.* 2010). Current therapeutic approaches, which include transsphenoidal resection, pharmacotherapy, and irradiation, remain inadequate in a significant number of patients (Daly *et al.* 2009, Galland *et al.* 2010). The degree of invasiveness appears to be a critical determinant for the success rate of surgical removal and thus for relapse. At present, the mechanisms that underlie the pathogenic processes of initiation, expansion, invasion, and/or relapse of pituitary tumors are largely unknown. Only a minority of the tumors (approximately 5%) are hereditary and can be traced back to genetic mutations; these exist either as

isolated tumors or as a component of a larger endocrine syndrome, like Men-1 (Daly *et al.* 2009, Melmed 2011).

In other types of tumors, so-called cancer stem cells (CSCs) have been identified. CSCs represent a pool of relatively undifferentiated transformed cells that self-renew and propagate the entire range of tumor cell progeny. Compared with the other cells in the tumor, the CSC subpopulation is considered to be more tumorigenic and more resistant to therapy, thereby surviving treatment and finally regrowing the (heterogeneous) tumor (Vankelecom & Gremeaux 2010, Clevers 2011, Vankelecom 2012, Vankelecom & Chen 2014). Recently, CSCs have been reported to display characteristics of epithelial–mesenchymal transition (EMT), which may drive these cells in their tumor expansion and invasive activity (Mani *et al.* 2008, Morel *et al.* 2008, Kong *et al.* 2010, Pirozzi *et al.* 2011, Yoon *et al.* 2012). EMT represents a multi-step cell-conversion process during which epithelial cells gradually acquire mesenchymal features. Cells change morphology, lose polarity and cell–cell contacts (e.g., by loss of E-cadherin), become mobile with enhanced invasive activity, and acquire increased resistance to apoptosis. In general, EMT is known to play a key role in cancer pathogenesis, particularly during the growth, maintenance (including resistance to hostile conditions), and progression of the tumor with local invasion and/or metastasis (De Craene & Berx 2013). In several types of cancer, it has been shown that EMT markers are enriched in the CSC fraction, that the induction of EMT causes the upregulated expression of 'stemness' genes, and that EMT acts as a key driver in the generation and activity of the CSCs (Mani *et al.* 2008, Morel *et al.* 2008, Kong *et al.* 2010, Pirozzi *et al.* 2011, Yoon *et al.* 2012).

Although the presence and functional (or clinical) relevance of CSCs has increasingly been documented in a variety of cancers (Clevers 2011, Eppert *et al.* 2011, Chen *et al.* 2012, Schepers *et al.* 2012, Boumahdi *et al.* 2014, Vanner *et al.* 2014, Zhu *et al.* 2014), the concept remains the subject of debate and is probably not applicable to all types of cancer. Nonetheless, the model may help us to understand the pathogenesis, therapy resistance, and

recurrence of tumors. At present, CSCs have mostly been identified in malignant types of cancer. Whether benign tumors also contain CSCs (in which case they would be better referred to as tumor stem cells (TSCs)) has only limitedly been studied (Vankelecom & Gremeaux 2010, Lapouge *et al.* 2011, Boumahdi *et al.* 2014). The search for CSCs is generally performed on the basis of 'stemness'-associated membrane markers or functional traits. High efflux capacity, as mediated by ATP-binding cassette (ABC) multidrug transporters, is considered to be one functional characteristic of CSCs; this property renders the cells more resistant to chemotherapeutic drugs. In the present study, we searched for cells that possess such efflux capacity using the 'side population' (SP) technique (Chen *et al.* 2005, 2009). Cells that extrude Hoechst dye are observed as a side branch of Hoechst^{low} cells in dual-wavelength fluorescence-activated cell sorting (FACS) analysis. In a variety of cancer types, a SP has been identified and found to be enriched in CSCs or CSC-like cells (Patrawala *et al.* 2005, Wu & Alman 2008, Kato *et al.* 2010, Britton *et al.* 2012, Moti *et al.* 2014). Thus, we searched for SP cells in human pituitary adenomas and in additional models of pituitary tumors (mouse tumors, cell lines, and xenograft tumors). We identified a SP and observed molecular and functional characteristics that are supportive of a TSC-associated phenotype. In addition, we identified the chemokine (C-X-C motif) receptor 4 (Cxcr4) pathway as an interesting target for further investigation in pituitary tumor treatment.

Materials and methods

Pituitary adenomas

Human pituitary adenoma samples were obtained immediately after transsphenoidal resection at the University Hospitals Leuven (Neurosurgery Division). Informed consent was obtained from the patients. Hormonal phenotype was determined by the Department of Imaging and Pathology (University Hospitals Leuven). An overview of the samples with clinical data is provided in Table 1. Sexes were represented approximately equally with an average age of 53 years (range 19–84 years) at the time of surgery. The majority of the tumors were NF-A ($n=34$) and GH-A ($n=21$). The other types of resected pituitary tumors were typically microadenomas, which only yielded sufficient study material in a limited number of cases (ACTH-A ($n=3$)) or were not surgically removed but were instead treated pharmacologically (resected PRL-A ($n=2$)).

Tumor samples were either immediately processed for flow cytometric analysis after resection, or they were cryopreserved as small pieces in Leibovitz-L-15 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Lonza, Rockland, ME, USA) and 10% DMSO (Sigma-Aldrich). They were then stored at -196°C for later examination. The freezing procedure did not change the SP proportion (Supplementary Fig. S1, see section on supplementary data given at the end of this article) or the expression characteristics, as was evident from the results of the microarray analyses.

Dissociation of human pituitary adenomas into single cells

Tumor samples were rinsed in DMEM (Life Technologies) supplemented with 0.3% BSA (Serva, Heidelberg, Germany) and incubated with collagenase type IV (1 mg/ml in Medium 199; Life Technologies) for 2–2.5 h at 37°C . Cell debris was removed by centrifugation through a 3% BSA layer. The resulting cell pellet was resuspended in pituitary-optimized, serum-free chemically defined medium (SFDM, Life Technologies; supplemented with 0.5% BSA; Chen *et al.* 2005, 2009) for further analyses.

Immunohistology of human pituitary adenoma sections

Formalin-fixed paraffin-embedded pituitary adenoma blocks were obtained from the Biobank (University Hospitals Leuven). Antigen retrieval was performed on $5\text{ }\mu\text{m}$ sections with EnVision FLEX Target Retrieval Solution (Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked with EnVision Peroxidase-Blocking Reagent, and sections were incubated with primary antibodies directed against human E-cadherin (ready-to-use; Dako), vimentin (VIM; 1:500; Dako), NOTCH2 (1:200; Abcam, Cambridge, UK), and interleukin 6 (IL6; 1:200; Abcam). Subsequently, samples were processed using EnVision Dual Link (Dako). The complex formed was visualized with 3,3'-diaminobenzidine (Dako), and sections were counterstained with hematoxylin (Dako).

Isolation and dissociation of mouse pituitaries

C57/Bl6 mice were purchased from Elevage Janvier (BioServices, Uden, The Netherlands), and dopamine receptor D2 knockout (*Drd2*^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept or bred in the KU Leuven Animal Facility under

Table 1 Summary of clinical and experimental data for the pituitary adenoma (PA) samples analyzed

PA sample number	Sex	Age at time of surgery (years)	PA phenotype	Size	Fresh or cryo-preserved	SP (percentage of total cells)	CD31 ⁺ (percentage of SP)	CD45 ⁺ (percentage of SP)	Microarray	Quantitative RT-PCR	IHH
PA 01	M	62	NF-A	Macro	F	7.5	ND	ND			
PA 02	M	41	NF-A	Macro	F	1.5	ND	ND			
PA 03	M	46	GH-A	Macro	F	1.8	ND	ND	x		
PA 04	M	37	GH-A	Macro	F	1.4	ND	ND			
PA 05	F	28	GH-A	Macro	F	1.8	ND	ND			
PA 06	M	84	GH-A	Macro	F	1.4	ND	ND	x		
PA 07	F	76	GH-A	Macro	F	1.6	ND	ND	x		
PA 08	M	39	NF-A	Micro	F	2.0	ND	ND			
PA 09	F	54	NF-A	Macro	F	2.7	ND	ND			
PA 10	M	59	GH-A	Macro	C	2.3	ND	ND	x		
PA 11	F	59	GH-A	Macro	C	8.0	ND	ND			
PA 12	M	71	GH-A	Macro	C	1.8	ND	ND			
PA 13	F	44	ACTH-A	Micro	F	0.5	ND	ND			
PA 14	F	72	NF-A	Macro	C	1.2	ND	ND			
PA 15	M	79	NF-A	Macro	C	1.4	ND	ND			
PA 16	M	72	GH-A	Micro	C	0.7	ND	ND			
PA 17	M	35	NF-A	Macro	C	0.5	ND	ND	x		
PA 18	F	43	GH-A	Micro	C	1.2	ND	ND			
PA 19	F	57	NF-A	Macro	F	17.2	ND	ND	x		
PA 20	F	64	NF-A	Macro	F	1.5	ND	ND	x		
PA 21	F	48	ACTH-A	Micro	F	1.6	ND	ND			
PA 22	F	77	NF-A	Macro	C	3.3	ND	ND	x		VIM ⁺ IL6 ⁻ NOTCH2 ⁻
PA 23	F	29	NF-A	Macro	F	0.6	ND	ND	x		VIM ⁺ IL6 ⁺ NOTCH2 ⁺
PA 24	F	60	GH-A	Macro	F	1.6	18.2	ND			
PA 25	F	19	NF-A	Macro	F	0.8	89.9	ND			
PA 26	M	43	NF-A	Macro	C	4.8	93.8	ND			
PA 27	M	40	NF-A	Macro	C	1.8	87.2	ND			
PA 28	F	34	GH-A	Macro	F	0.8	38.9	ND			
PA 29	F	54	NF-A	Macro	C	1.5	93.8	ND			
PA 30	F	42	NF-A	Macro	F	1.3	70	7.9			
PA 31	M	76	NF-A	Macro	C	0.7	84	3.4			
PA 32	M	62	GH-A	Macro	F	1.3	81.6	4.8			
PA 33	M	80	NF-A	Macro	C	0.6	23.6	1.7	x	x	
PA 34	M	70	NF-A	Macro	F	1.3	24	27.3	x	x	VIM ⁺ IL6 ⁻ NOTCH2 ⁺
PA 35	M	40	NF-A	Macro	C	1.5	18.7	11.6	x	x	VIM ⁻ IL6 ⁻ NOTCH2 ⁻
PA 36	M	62	NF-A	Macro	C	0.6	71.6	17.8			
PA 37	F	50	GH-A	Micro	C	1.8	57.5	6.7			
PA 38	M	70	NF-A	Macro	C	0.6	60.9	3.9	x	x	VIM ⁺ IL6 ⁺ NOTCH2 ⁺
PA 39	F	39	PRL-A	Macro	C	1.2	43.3	33.5			
PA 40	M	63	NF-A	Macro	F	1.1	12.6	66.6	x	x	VIM ⁺ IL6 ⁺ NOTCH2 ⁺
PA 41	M	39	NF-A	Macro	F	0.9	22	3.9			
PA 42	F	30	NF-A	Macro	C	0.5	13	40.4			
PA 43	M	61	GH-A	Macro	C	6.8	95.2	1.3			
PA 44	M	49	NF-A	Macro	F	0.6	33	35		x	
PA 45	M	63	NF-A	Macro	F	2.4	60	4			
PA 46	F	27	GH-A	Macro	C	0.6	23	33.5		x	

Table 1 Continued

PA sample number	Sex	Age at time of surgery (years)	PA phenotype	Size	Fresh or cryo-preserved	SP (percentage of total cells)	CD31 ⁺ (percentage of SP)	CD45 ⁺ (percentage of SP)	Microarray	Quantitative RT-PCR	IHH
PA 47	F	44	ACTH-A	Micro	F	1.5	5.8	37.3			
PA 48	F	39	NF-A	Macro	F	1.2	43.3	33.5			
PA 49	M	66	NF-A	Macro	C	0.4	80.7	2.4			
PA 50	F	28	PRL-A	Macro	C	1.4	75.5	4.6			
PA 51	F	77	GH-A	Macro	C	0.5	12.7	0.2			
PA 52	F	74	NF-A	Macro	C	1.7	81.5	5.5			
PA 53	F	62	GH-A	Macro	C	1.5	5.8	37.3			
PA 54	F	62	GH-A	Macro	C	1.9	29.7	23			
PA 55	M	62	NF-A	Macro	C	0.6	63.6	1.4			
PA 56	M	46	NF-A	Micro	C	1.6	75.9	1.1			
PA 57	M	45	GH-A	Macro	C	1.9	29.7	23			
PA 58	M	46	GH-A	Macro	C	1.9	29.7	23			
PA 59	M	52	NF-A	Macro	C	0.8	86	4.3			
PA 60	M	65	NF-A	Macro	C	0.5	48.4	1.1			

ND, not determined; IHH, immunohistochemistry; NF-A, non-functioning adenoma; GH-A, somatotropinoma; ACTH-A, corticotropinoma; PRL-A, prolactinoma; micro, micro-adenoma: <1 cm; macro, macro-adenoma: ≥1 cm.

conditions of constant temperature, humidity, and day–night cycle in a sterile environment. The mice were allowed to consume food and water *ad libitum*. Animal experiments were approved by the KU Leuven Ethical Committee.

The pituitary was isolated from killed mice as previously described (Chen *et al.* 2005, 2009). The anterior lobes (anterior pituitary (AP)) of the *Drd2*^{−/−} mice that grow prolactinomas (from 6 to 14 months of age; Cristina *et al.* 2006) and those of the respective control mice (*Drd2*^{+/-} and *Drd2*^{+/+}) were dispersed into single cells using collagenase type IV as explained in the previous sections for human pituitary adenomas.

Culture and treatment of AtT20

The mouse corticotrope AtT20 cell line was obtained from the American Type Culture Collection (ATCC, CCL-89; Manassas, VA, USA). The cell line was authenticated by hormone expression analysis with quantitative RT-PCR (qRT-PCR; see the subsequent section on qRT-PCR). The cells were cultured in DMEM/F12 (Life Technologies) supplemented with 10% FBS. To investigate the involvement of the transforming growth factor beta (TGFβ) or Cxcr4 pathways, AtT20 cells were cultured in serum-free medium for 18–24 h and were then treated with TGFβ1 (5 ng/ml; R&D Systems, Minneapolis, MN, USA) or AMD3100 (octahydrochloride, 100 ng/ml; Sigma–Aldrich) respectively. Medium was refreshed every 24 h. To analyze gene expression and SP proportion after treatment (see the

subsequent sections on these methods), cells were detached and dissociated using trypsin (0.05%) with EDTA (0.02%; Life Technologies).

Flow cytometry

Dissociated cells (from human adenoma or mouse AP, AtT20 cell line, or xenograft tumors – see subsequent sections) were processed for SP analysis as described in detail previously (Chen *et al.* 2005, 2009). In short, cells were incubated with the vital dye Hoechst 33342 (Sigma–Aldrich) at a final concentration of 2.5 µg/ml (for mouse AP and AtT20) or 5 µg/ml (for adenoma and xenograft samples). Subsequently, cells were either immediately analyzed by FACS (Vantage or Aria III; BD Biosciences, Erembodegem, Belgium) or they were first stained with phycoerythrin (PE)-conjugated mouse anti-human CD45, FITC-conjugated mouse anti-human CD31, PE-conjugated mouse anti-mouse CD45, and/or FITC-conjugated rat anti-mouse CD31 antibodies using dilutions recommended by the manufacturer (BD Biosciences). Samples were examined for SP on the basis of dual-wavelength emission using appropriate filters (Vantage: 424/44 and 630/22; Aria III: 450/40 and 630/20) after (near-)u.v. excitation (360 and 375 nm on Vantage and Aria III respectively). The SP phenotype was verified by adding verapamil (100 µM; Sigma–Aldrich), which blocks the Hoechst efflux. The bulk cell population that did not expel Hoechst was designated as the ‘main population’ (MP). CD31 and CD45 staining was controlled using isotype antibodies (BD Biosciences; data not shown).

Xenograft tumors and *in vivo* tumorigenic activity

In an attempt to grow human pituitary adenoma in immunodeficient mice, tumor pieces or dissociated cells ($1\text{--}1.5 \times 10^6$) were transplanted s.c. (GH-A and NF-A ($n=8$)) or under the kidney capsule (GH-A and ACTH-A ($n=3$)). Male mice (6–10 weeks old) with increasing grades of immunodeficiency (from SCID to NOD-SCID to NOD-SCID^{IL2Rγ^{-/-}}) were purchased from Elevage Janvier and were anesthetized with Avertin (tribromoethanol; Sigma-Aldrich). Before injection, cells were resuspended in DMEM mixed with Matrigel (1:1, BD Biosciences). Three to six months later, the mice were killed and examined for tumor growth. Tissue from the implantation sites was dissected, and hematoxylin and eosin staining was performed.

To grow xenograft tumors from AtT20 cells, SCID mice (males; 6–10 weeks old) received s.c. injections of 5×10^5 cells. To assess the tumorigenic (TSC) activity of the AtT20 SP versus the MP, cell populations were sorted into DMEM/F12/10% FBS, mixed with Matrigel (1:1) and s.c. injected at different cell numbers into SCID mice (SP cells into one flank and a similar number of MP cells into the other flank of the same mouse). Tumor size was measured using a caliper, and volume was calculated with the following formula: $((\text{large side}) \times (\text{small side})^2) \times 0.52$.

To explore the involvement of the Cxcr4 pathway, AtT20 xenograft tumors that developed in SCID mice were treated with AMD3100 by intratumoral injection (5 μg, two times per week) starting from day 21 after the s.c. AtT20 cell implantation.

Tumorsphere- and colony-forming assay

Dissociated human pituitary adenoma cells were seeded at a density of 100 000 cells/ml into 35 mm nontreated culture dishes (Iwaki, Scitech, Chiba, Japan) and cultured in SFDM supplemented with B27 (1:50; Life Technologies) and recombinant basic fibroblast growth factor (bFGF, 20 ng/ml; R&D Systems). Fresh medium was added every 3 days. To assess their self-renewal capacity, the spheres were harvested and dissociated into single cells using trypsin (Chen *et al.* 2009), and the cells were reseeded under similar conditions.

To evaluate their colony-forming capacity, cells were cultured at low density (1000–4000 cells/60 mm dish) in Ultraculture medium (Life Technologies) to which 5% FBS, 20 ng/ml bFGF, and 50 ng/ml cholera toxin (Sigma-Aldrich) were added. Medium was changed every 3 days. Cultures were fixed with methanol and stained with

1,9-dimethyl-methylene blue (Sigma-Aldrich), and the number of colonies was determined. Live pictures of the formed spheres and colonies were taken using a Nikon Eclipse TS100 microscope (Nikon Instruments, Melville, NY, USA).

Cell motility assay

Cell motility was evaluated using the ‘scratch assay’. After reaching 90% confluence, AtT20 cells were serum-starved for 24 h and then treated with mitomycin C (10 μg/ml; Kyowa, Tokyo, Japan) to inhibit cell proliferation. A straight scratch was created, and cells were further kept in DMEM/F12, together with TGFβ1 (5 ng/ml), AMD3100 (100 ng/ml), or vehicle. Medium was changed every 24 h. The migration of cells into the scratch was evaluated by light microscopy, and live pictures were taken with the Nikon Eclipse TS100 microscope at different time points. The open area was calculated using analysis-algorithm-based TScratch Software (CSElab-ETH, Zurich, Switzerland).

Small-animal magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed on *Drd2^{-/-}* and *Drd2^{+/-}* mice using a horizontal 9.4-Tesla nuclear magnetic resonance spectrometer and a 40 mm-diameter birdcage coil as the radio frequency transmitter and receiver. Mice were anesthetized using 1% isoflurane in a mixture of 75% air and 25% oxygen. T1-weighted images were acquired using a two-dimensional multiple-slice spin echo sequence, with an echo time of 11 ms, a repetition time of 500 ms, an in-plane resolution of 0.1×0.1 mm, 24 coronal slices with 0.3 mm slice thickness, and ten signal averages. Image reconstruction was performed using the spectrometer console. Ten minutes before the scanning procedure, mice received an i.p. injection of the contrast agent gadolinium (0.50 mmol/kg body weight).

Immunofluorescence analysis

For the immunofluorescence examination of mouse pituitaries, whole glands were isolated and fixed with paraformaldehyde (4%; Riedel-de Haën, Seelze, Germany). After agarose embedding, pituitaries were coronally sectioned into 45 μm slices using a vibratome (Microm HM 650 V; Prosan, Merelbeke, Belgium). Sections were permeabilized with 0.4% Triton X-100 (Sigma-Aldrich) and incubated overnight with the following primary antibodies: goat anti-human SOX2 (1:750; Immune

Systems, Devon, UK) and rabbit anti-mouse PRL (1:10 000; from Dr A F Parlow, NHPP, Harbor-UCLA Medical Center, Torrance, CA, USA). Finally, sections were incubated with AlexaFluor 488-conjugated donkey anti-rabbit and/or AlexaFluor 555-conjugated donkey anti-goat antibodies (1:1000; Life Technologies), and nuclei were labeled with ToPro3 (Life Technologies). Immunofluorescence was examined with a Zeiss LSM 510 confocal laser-scanning microscope (Zeiss, Zaventem, Belgium) accessible through the Cell Imaging Core (CIC; KU Leuven). Z-stacks were collected and analyzed with the Zeiss LSM Image Browser and ImageJ (<http://imagej.nih.gov/ij/>), and pictures were prepared using the same programs and Microsoft PowerPoint (2007).

Immunofluorescence staining of paraffin-embedded mouse pituitary sections (5 μ m) was performed after antigen retrieval with citrate buffer (pH 6) and permeabilization with Triton X-100 (0.1% in PBS). Following incubation with primary and secondary antibodies (as described earlier), sections were covered with Vectashield (containing 4',6-diamidino-2-phenylindole (DAPI)) and analyzed with a Leica DM5500 epifluorescence microscope (Leica Microsystems, Diegem, Belgium) accessible through InfraMouse (KU Leuven-VIB). Recorded images were converted to pictures with ImageJ and Microsoft PowerPoint (2007).

Cytospin samples of dissociated mouse AP cells were fixed with paraformaldehyde (4%), permeabilized with saponin (0.5%; Sigma-Aldrich), and further immunostained for Sox2 (with goat anti-human SOX2 at 1:250; Immune Systems) as described in the previous sections. In some of the analyses, cells were simultaneously immunostained for the proliferation marker Ki67 (with rabbit anti-Ki67 at 1:50; Thermo Scientific, Fremont, CA, USA). Nuclei were counterstained with DAPI (0.5 μ g/ml). To enumerate Sox2-immunopositive cells, pictures were captured using a Leica DM5500 microscope, and cells were counted with ImageJ in five to ten random fields per group in each experiment (about 1500 cells/group in each experiment). The proportions of the Sox2⁺ cells (of total cells counted) were determined. Because of the increase in total AP cells in the *Drd2*^{-/-} adenomatous pituitary, comparison of the proportions is not appropriate. Thus, the absolute numbers of Sox2⁺ cells were calculated (using their proportion and the total number of AP cells obtained per experimental group of mice) for comparisons between *Drd2*^{-/-}, *Drd2*^{+/-}, and *Drd2*^{+/+} mice.

For the immunofluorescence examination of tumor-spheres developed from human pituitary adenomas, cells were fixed with paraformaldehyde (4%), permeabilized

with Triton X-100 (0.4% in PBS), and incubated with goat anti-human SOX2 (1:250; Immune Systems), rat anti-mouse nestin (1:500; Abcam), and/or rabbit anti-human GH (for spheres originating from GH-A; 1:2000; Dako). Secondary antibodies included AlexaFluor 555-conjugated donkey anti-goat, AlexaFluor 488-conjugated donkey anti-rat, and AlexaFluor 488-conjugated donkey anti-rabbit (all at 1:1000; Life Technologies). Pictures were obtained using a Leica DM5500 epifluorescence microscope as described in the previous sections.

AtT20 cell death, apoptosis, and proliferation

The number of dying or dead AtT20 cells after AMD3100 treatment (100 ng/ml) was determined after staining with Trypan blue (0.4%; Sigma-Aldrich). Apoptosis was scored by TUNEL analysis in cytospin samples of AtT20 cells from culture or xenograft tumors using the FragEL Kit (Calbiochem, Darmstadt, Germany). Cell proliferation was quantified by the immunofluorescence staining of cytospin samples for Ki67 as described earlier in the present study. Pictures were taken and cells enumerated (see previous sections).

Whole-genome expression profiling

Total SP and MP cells (NF-A ($n=5$) and GH-A ($n=4$)) or CD31⁻/CD45⁻ SP (purified SP (pSP)) and pMP cells (NF-A ($n=5$)) were sorted by FACS into cold lysis solution of the RNeasy Micro Kit (Qiagen). Total RNA was extracted immediately after cell collection in accordance with the manufacturer's guidelines. RNA samples were stored at -80°C until further processing for whole-genome expression profiling (in collaboration with the VIB Nucleomics Core, KU Leuven). RNA quality and concentration were determined with Agilent Picochips on an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Two nanograms of high-quality RNA (with RNA integrity number ≥ 8.0) was amplified using the NuGen Pico WTA Kit (NuGen Technologies, San Carlos, CA, USA). Cy3 label was incorporated into the cRNA probes, which were then hybridized onto Agilent whole-human genome 44K oligonucleotide arrays. Slides were scanned and data were processed using Agilent's Feature Extraction Software for background correction, quantile normalization, principal component analysis (PCA), hierarchical clustering, and MA and volcano plots. Microarray data are available from the NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/projects/geo/>) through the series accession number GSE62960.

To determine differentially expressed genes (probes), a stringent analysis was initially applied to the normalized data sets. Taking into account that the data are paired (SP versus MP for each sample), analysis was based on the $\log_2$ ratios computed for each pair. $\log_2$ ratios were compared with the Linear Models for Microarray Data (Limma) package of Bioconductor in R, and a moderated t -statistic (implemented in Limma) was performed to test the significance of the obtained differences. For the first microarray data set (total SP versus MP), the resulting P values were corrected for multiple testing with Benjamini–Hochberg to control the false discovery rate. Differentially expressed genes were withheld when the corrected P value was lower than 0.05. For the second microarray data set (CD31[−]/CD45[−] SP versus CD31[−]/CD45[−] MP), Benjamini–Hochberg could not be applied because of the low number of samples. Therefore, a cutoff based on the uncorrected P values (<0.001) was utilized. For both data sets, the cutoff for the P values was combined with a cutoff for the fold change (≥ 2) to finally identify the differentially expressed genes.

Given the rather small number of adenomas analyzed thus far, which causes a very high cutoff for differential expression and hence the discarding of potentially interesting differences, we additionally applied a less stringent analysis by performing paired Student's t -tests on the individual probe sets. Genes were judged to be differentially expressed when $P < 0.05$ and fold change ≥ 1.5 . We further considered genes that showed a significant expression difference in five to eight out of the nine adenomas analyzed for total SP and MP, and in two to four out of the five adenomas examined for CD31[−]/CD45[−] fractions.

Functional clustering analysis of differentially expressed genes (gene ontology (GO)) was performed using the Database for Annotation, Visualization and Integrated Discovery version 6.7 (DAVID, www.david.abcc.ncifcrf.gov). Gene clusters with an enrichment score of > 1.5 ($-\log$ of the median P value for the functional group) were retained. Finally, we narrowed the gene sets by focusing on factors that have been shown to be important in CSC/'tumor stemness', including EMT, NOTCH, WNT/ β -CATENIN, and TGF β /BMP.

Gene expression analysis by qRT-PCR

Amplified cDNA of the CD31[−]/CD45[−] SP and MP fractions from the five microarrayed NF-A samples, as well as that from two additional samples (NF-A ($n=1$) and GH-A ($n=1$); see Table 1), was examined by qRT-PCR to validate the expression of a selection of genes with Fast

SYBR Green Master Mix and an ABI 7900HT Fast RT-PCR system according to the manufacturer's instructions (Life Technologies).

Gene expression analysis of AtT20 cells and dispersed xenograft tumors was performed using a similar approach (but without the RNA amplification step). cDNA was subjected to qPCR in a LightCycler 480 with the LightCycler FastStart DNA Master Plus SYBR Green I (Roche).

Forward and reverse primers were designed using PerlPrimer (<http://perlprimer.sourceforge.net>) or were obtained from a public database (<http://medgen.ugent.be/rtpriprimerdb>), and they are described in Supplementary Table S1, see section on supplementary data given at the end of this article. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β 2-microglobulin (*B2M*), and/or hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were used to normalize gene expression. Their expression was found to be stable (data not shown). Relative gene expression levels were calculated as change in the threshold cycle (ΔC_t) values (' C_t target $- C_t$ reference') and were compared between the SP and MP as fold change ($2^{-\Delta C_t \text{ SP}} / 2^{-\Delta C_t \text{ MP}}$). Statistical analysis was performed using GraphPad Prism version 5.2 (GraphPad Software, La Jolla, CA, USA) by applying Mann–Whitney U tests. P values of < 0.05 were considered statistically significant.

For authentication of the AtT20 cell line used, hormone gene expression was examined by qRT-PCR. The corticotrope phenotype of the cell line was confirmed by the detection of proopiomelanocortin (*POMC*; a precursor of ACTH) expression, whereas *GH* and *TSH β* gene transcription was not found (see Supplementary Table S2, see section on supplementary data given at the end of this article).

Results

Human pituitary tumors contain a SP

Human pituitary adenomas ($n=60$; see Table 1) were examined for the presence of a SP. In all of the tumors analyzed, a Hoechst^{low} cell population was detected that was no longer observed when the efflux-blocker verapamil was added together with Hoechst (Fig. 1A), thereby demonstrating the SP phenotype (mean SP $\pm$ S.E.M. $1.9 \pm 0.3\%$ of the total viable tumor cells; range 0.4–17.2%; Table 1).

SP proportions were not different between the samples analyzed immediately after surgical resection ($n=26$) or those analyzed after cryopreservation ($n=34$) (Table 1 and Supplementary Fig. S1A, see section on supplementary data given at the end of this article) or between the

Figure 1

Identification and characterization of the side population (SP) in human pituitary adenomas. (A) Dot plots of dual-wavelength FACS analysis of human pituitary adenoma cells after incubation with Hoechst 33342 alone (left) or together with verapamil (right). The SP (black) and MP (white) are gated, and SP proportions are indicated. A representative example is shown. (B) Boxplot of SP proportions obtained from NF-A ($n=33$) and GH-A ($n=20$) as well as from all samples taken together ($n=60$). Minimum and maximum values (whiskers) and outliers (dots) are indicated. (C) Dot plots of FACS analysis of human pituitary adenoma cells after incubation with

Hoechst 33342 and immunostaining for CD31 and CD45. The CD31⁺, CD45⁺, and CD31⁻/CD45⁻ cells within the SP are also shown (inset). (D) Percentage of CD31⁺ (upper panel) and CD45⁺ cells (lower panel) within the SP versus the SP proportion within the pituitary adenomas analyzed ($n=37$ and $n=31$ respectively). (E) Expression analysis by quantitative RT-PCR of a selection of markers identified as differentially expressed in the pituitary adenoma CD31⁻/CD45⁻ SP (pSP) versus the pMP by microarray analysis. Bars indicate means of fold expression pSP/pMP $\pm$ S.E.M. ($n=7$ pituitary adenomas). * $P<0.05$.

different hormonal phenotypes examined (Table 1 and Fig. 1B). The adenoma SP predominantly clusters cells of small to medium size (low 'forward scatter') and low granularity (low 'side scatter') (Supplementary Fig. S1B).

Whole-genome expression profiling of the adenoma SP points to EMT and 'tumor stemness'

SP cells and the tumor bulk MP cells from somatotropinomas (GH-A ($n=4$)) and NF-A ($n=5$) (see Table 1) were sorted by FACS and subjected to gene expression profiling using whole-genome microarrays. PCA indicated a similar segregation of the SPs from the corresponding MPs for the majority of the samples analyzed (Supplementary Fig. S1C). Hierarchical clustering of differentially expressed genes also largely separated the SPs from the MPs (Supplementary Fig. S1D).

The set of genes that was most differentially expressed ($P<0.001$; 2760 probe sets) was submitted to DAVID for GO analysis, which yielded a total of 1688 corresponding human ENSEMBL IDs. Gene set enrichment exposed in the SP the upregulation of biological functions that are involved in cell proliferation, vascular development, cell migration and motility, and lymphocyte activation (Supplementary Table S3, see section on supplementary data given at the end of this article).

A focused analysis of a selection of genes using the expression data of the nine adenomas (Table 2 and Supplementary Table S4, see section on supplementary data given at the end of this article) revealed upregulated expression (i.e., >1.5 -fold in at least two-thirds, or in five, of the nine tumors, $P<0.05$; see the 'Materials and methods' section) in the SP of the multidrug transporters *ABCB1* and *ABCG2*, which typically underlie the SP phenotype. This finding therefore validates the SP identification and sorting procedure. Remarkably, the transcriptome displayed an expression pattern that supports the occurrence of EMT in the SP compartment. The epithelial markers E-cadherin (*CDH1*) and claudin-1 (*CLDN1*) were downregulated in the pituitary adenoma SP, whereas the mesenchymal markers *VIM* and fibronectin 1 (*FN1*) were upregulated (Table 2 and Supplementary Table S4). Moreover, recognized markers of EMT, such as *SNAIL*, *ZEB1*, and *ZEB2* (all of which are transcriptional repressors of the *CDH1* gene), were more highly expressed in the SP vs the MP, together with other inducers and regulators of EMT (*KLF8*, *LEF1*, *TCF4*, *FOXC1*, *HOXB9*, *FOSL2*, *MMP1/2*, and *CXCR4*). Some of the pathways that were identified as being upregulated are also well known for their regulatory input in EMT (WNT, TGF β , epiregulin,

and amphiregulin). In the present analysis, we also observed that factors often associated with 'tumor stemness' or CSCs, including *CD44*, *CXCR4*, *KIT*, *KLF4*, and *NESTIN* were up-regulated in the SP. In addition, the signaling pathways of NOTCH, WNT, and TGF β -BMP have also been related to 'tumor stemness'. Overall, no obvious differences were observed between the SP transcriptomes of the NF-A and the GH-A samples.

Notably, the microarray analysis also revealed the upregulated expression of genes associated with an endothelial phenotype (such as *PECAM1/CD31*, *VWF*, *CDH5*, *VCAM1*, *FLK1/KDR*, *TIE1*, and *TEK/TIE2*) or an immune character (such as *CD45/PTPRC*) in the adenoma SP (see Table 2 and Supplementary Table S4). These expression characteristics are consistent with the enriched biological processes described earlier (viz., vascular development and lymphocyte activation; see Supplementary Table S3), and they indicate the presence of endothelial and immune/hematopoietic cells in the SP. Previously, we and others discovered that the SP, either from healthy tissue or tumors, represents a still heterogeneous population that segregates not only cells with (tumor) stem cell properties but also some other cells of endothelial and immune/hematopoietic phenotype (Pfister et al. 2005, Uezumi et al. 2006, Chen et al. 2009).

Taken together, our microarray expression data indicate that, as compared with the MP, the pituitary adenoma SP is enriched in cells with EMT- and 'tumor stemness'-associated molecular traits, but it is still heterogeneous in cell phenotypes.

EMT- and 'tumor stemness'-related expression characteristics remain present in the adenoma SP depleted from endothelial and immune cells

The pituitary adenoma SP was then analyzed for the presence of endothelial (CD31⁺) and immune/hematopoietic (CD45⁺) cells. Flow cytometry revealed a CD31⁺ cell population of varying abundance in the SP (mean $\pm$ S.E.M. 50.9 $\pm$ 4.8% of the SP; range 5.8–95.2%; $n=37$; Fig. 1C and D; Table 1). Also, CD45⁺ cells were found, but in general they constituted a lower fraction of the adenoma SP (mean $\pm$ S.E.M. 16.2 $\pm$ 3.0% of the SP; range 0.2–66.6%; $n=31$; Fig. 1C and D; Table 1). The non-endothelial, non-immune (CD31[−]/CD45[−]) SP fraction finally comprised on average one-third of the total SP (mean $\pm$ S.E.M. 32.3 $\pm$ 2.2%; range 7.2–64.1%), 0.5% ($\pm$ 0.1) of all the CD31[−]/CD45[−] adenoma cells and 0.1% ($\pm$ 0.01) of the total tumor cell population. In general, no correlation was found between the size of the total SP

Table 2 Expression of a selection of genes in the total SP of human pituitary adenomas as determined from the results of microarray analysis

Gene ^a	Gene description	Expression in the SP ^b
(Tumor) stemness		
<i>ABCB1</i> (<i>MDR1/TAP</i>)	ATP-binding cassette, sub-family B (<i>MDR/TAP</i>), member 1	A
<i>ABCG2</i> (<i>BCRP1</i>)	ATP-binding cassette, sub-family G (<i>WHITE</i>), member 2 (junior blood group)	A
<i>CD44</i>	CD44 molecule (Indian blood group)	B
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4	A
<i>KIT</i>	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	A
<i>KLF4</i>	Kruppel-like factor 4 (gut)	A
<i>NES</i>	Nestin	A
Notch pathway (and related)		
<i>DLL1</i>	Delta-like 1 (<i>Drosophila</i>)	A
<i>DLL3</i>	Delta-like 3 (<i>Drosophila</i>)	C
<i>DLL4</i>	Delta-like 4 (<i>Drosophila</i>)	A
<i>HES1</i>	Hes family bHLH transcription factor 1	A
<i>HES2</i>	Hes family bHLH transcription factor 2	A
<i>HES5</i>	Hes family bHLH transcription factor 5	A
<i>HEYL</i>	Hes-related family bHLH transcription factor with YRPW motif-like	A
<i>JAG1</i>	Jagged 1	A
<i>NOTCH1</i>	Notch 1	A
<i>NOTCH2</i>	Notch 2	B
<i>NOTCH4</i>	Notch 4	A
TGF β /BMP pathway (and related)		
<i>BMP2</i>	Bone morphogenetic protein 2	A
<i>BMP4</i>	Bone morphogenetic protein 4	A
<i>BMP6</i>	Bone morphogenetic protein 6	A
<i>MEN1</i>	Multiple endocrine neoplasia 1	D
<i>SMAD2</i>	SMAD family member 2	D
<i>SMAD3</i>	SMAD family member 3	A
<i>SMAD4</i>	SMAD family member 4	D
<i>TGFB1</i>	Transforming growth factor, β 1	A
<i>TGFB2</i>	Transforming growth factor, β 2	D
<i>TGFB2R2</i>	Transforming growth factor, β receptor 2 (70/80 kDa)	A
<i>TGFB2R3</i>	Transforming growth factor, β receptor 3	A
WNT/ β -catenin pathway (and related)		
<i>APC</i>	Adenomatous polyposis coli	D
<i>AXIN2</i>	Axin 2	D
<i>CD44</i>	CD44 molecule (Indian blood group)	B
<i>CDH1</i>	Cadherin 1, type 1, E-cadherin (epithelial)	C
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1, 88 kDa	A
<i>DKK1</i>	Dickkopf WNT signaling pathway inhibitor 1	A
<i>DKK2</i>	Dickkopf WNT signaling pathway inhibitor 2	A
<i>FZD3</i>	Frizzled class receptor 3	D
<i>FZD4</i>	Frizzled class receptor 4	A
<i>FZD8</i>	Frizzled class receptor 8	A
<i>LRP5</i>	LDL receptor-related protein 5	A
<i>LEF1</i>	Lymphoid enhancer-binding factor 1	A
<i>MYC</i>	V-myc myelocytomatosis viral oncogene homolog	A
<i>WNT9B</i>	Wingless-type MMTV integration site family, member 9B	D
EMT		
Upregulated in EMT		
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4	A
<i>FN1</i>	Fibronectin 1	A
<i>FOSL2</i>	FOS-like antigen 2	B
<i>FOXC1</i>	Forkhead box C1	A
<i>HOXB9</i>	Homeobox B9	A
<i>KLF8</i>	Kruppel-like factor 8	A
<i>MMP1</i>	Matrix metalloproteinase 1	B
<i>MMP2</i>	Matrix metalloproteinase 2	B
<i>SNAI1</i>	Snail family zinc finger 1	A
<i>TCF4</i>	Transcription factor 4	A
<i>VIM</i>	Vimentin	A
<i>ZEB1</i>	Zinc finger E-box binding homeobox 1	B

Table 2 Continued

Gene ^a	Gene description	Expression in the SP ^b
<i>ZEB2</i>	Zinc finger E-box binding homeobox 2	A
Downregulated in EMT		
<i>CDH1</i>	Cadherin 1, type 1, E-cadherin (epithelial)	C
<i>CLDN1</i>	Claudin 1	C
Angiogenesis/endothelial		
<i>CDH5</i>	Cadherin 5, type 2 (vascular endothelium)	A
<i>FLK1/KDR</i>	Kinase insert domain receptor	A
<i>PECAM1 (CD31)</i>	Platelet/endothelial cell adhesion molecule 1	A
<i>TEK/TIE2</i>	TEK tyrosine kinase, endothelial	A
<i>TIE1</i>	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	A
<i>VCAM1</i>	Vascular cell adhesion molecule 1	A
<i>VWF</i>	von Willebrand factor	A
Immune/hematopoietic		
<i>CD45/PTPRC</i>	Protein tyrosine phosphatase, receptor type, C	B

^aSelection of genes as analyzed by microarray; genes are listed alphabetically per group.

^bExpression level of the gene in the total adenoma SP: A, when more than 1.5-fold upregulated in the SP versus the MP in at least two-thirds of the adenoma samples analyzed (six or more out of the nine); B, when more than 1.5-fold upregulated in the SP versus the MP in five out of the nine adenoma samples; C, when more than 1.5-fold downregulated in the SP versus the MP in at least six out of the nine adenoma samples analyzed; and D, when there is less than 1.5-fold difference versus the MP.

and the proportion of CD31⁺ or CD45⁺ cells (Table 1 and Fig. 1D), although the two samples with the highest SP proportion analyzed for these antigens (PA 26, and PA 43) contained the largest fractions of CD31⁺ cells in the SP.

To determine the whole-genome expression profiles of the non-endothelial, non-immune cell populations, SP and MP were depleted from CD31⁺/CD45⁺ cells during flow cytometric sorting (see Fig. 1C for gating; NF-A (*n*=5); Table 1). Stringent analysis revealed that 101 genes (probe sets), which correspond to 74 human ENSEMBL IDs in DAVID, were differentially expressed between the CD31[−]/CD45[−] SP (pSP) and the CD31[−]/CD45[−] MP (pMP). Eighty of these were upregulated in the pSP, and 21 were downregulated (Supplementary Fig. S2A, B and C, see section on supplementary data given at the end of this article). The relatively small number of samples analyzed thus far caused a high cutoff for differential expression (as is clear in Supplementary Fig. S2A). Therefore, we performed an additional, less stringent examination (see the 'Materials and methods' section) by analyzing genes that were altered (≥ 1.5 -fold, $P < 0.05$) not only in all of the adenomas but also in four out of the five tumors profiled. A total of 2374 genes (probe sets) were found to be differentially expressed in the pSP as compared with the pMP in at least four of the adenoma samples (corresponding to 1576 human ENSEMBL IDs), with 1662 and 712 genes being upregulated and downregulated respectively (Supplementary Table S5, see section on supplementary data given at the end of this article). The gene sets obtained in both the stringent and less stringent analyses

(101 and 2374 genes respectively) were submitted to GO examination using DAVID. Overviews of the most significantly enriched biological processes are provided in Supplementary Tables S6 and S7, and they include, among others, cell motility and migration, vascular development, cell proliferation, and organ development.

Subsequent analysis of the pSP was focused on a selection of genes as described earlier for the total SP, including genes that are associated with EMT and 'tumor stemness' (Table 3 and Supplementary Table S8, see section on supplementary data given at the end of this article). On the whole, the expression picture of the total SP appeared to be retrieved in the CD31[−]/CD45[−] SP, viz. The upregulated expression of *ABCG2* and of other CSC-associated markers, including *CD44*, *CD34*, *LIFR*, and *CXCR4*. *KIT* expression was upregulated in four of the five samples, and *NESTIN* was upregulated in three of the five (Table 3). In particular, the gene expression picture that underlies EMT was also present in the pSP, showing elevated expression of the mesenchymal markers *VIM* and *FN1* and of several transcriptional EMT regulators (including *SNAIL1*, *SNAIL2*, *ZEB1*, and *ZEB2*; Table 3). Because GO enrichment indicated gene clusters involved in cell motility and migration, and because EMT is known to drive the motile and invasive behavior of cells, additional genes involved in these processes were examined. A considerable number were found to be upregulated or downregulated in the pSP versus the pMP, including *EPHB1* and *TM4SF1* (Supplementary Table S8). Interestingly, epidermal growth factor receptor (*EGFR*) was also highly (ninefold) upregulated in

Table 3 Expression of a selection of genes in the pSP of human pituitary adenomas as determined from the results of microarray analysis

Gene ^a	Gene description	Expression in the pSP ^b
(Tumor) stemness		
<i>ABCG2 (BCRP1)</i>	ATP-binding cassette, sub-family G (WHITE), member 2 (junior blood group)	B
<i>CD34</i>	CD34 molecule	A
<i>CD44</i>	CD44 molecule (Indian blood group)	A
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4	A
<i>KIT</i>	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	B
<i>LIFR</i>	Leukemia inhibitory factor receptor α	A
<i>NES^c</i>	Nestin	C
Notch pathway (and related)		
<i>DLK1^c</i>	Delta-like 1 homolog (<i>Drosophila</i>)	D
<i>DLL1^c</i>	Delta-like 1 (<i>Drosophila</i>)	C
<i>DLL3^c</i>	Delta-like 3 (<i>Drosophila</i>)	D
<i>DLL4^c</i>	Delta-like 4 (<i>Drosophila</i>)	D
<i>HES1^c</i>	Hes family bHLH transcription factor 1	B
<i>HEY2^c</i>	Hes-related family bHLH transcription factor with YRPW motif 2	B
<i>JAG1^c</i>	Jagged 1	B
<i>JAG2^c</i>	Jagged 2	F
<i>NOTCH1^c</i>	Notch 1	C
<i>NOTCH2</i>	Notch 2	A
<i>NOTCH4^c</i>	Notch 4	D
TGF β /BMP pathway (and related)		
<i>BMP2</i>	Bone morphogenetic protein 2	A
<i>BMPR2</i>	Bone morphogenetic protein receptor, type 2 (serine/threonine kinase)	B
<i>TGFB1^c</i>	Transforming growth factor, β 1	A
<i>TGFB2</i>	Transforming growth factor, β 2	D
<i>TGFB1</i>	Transcription factor, β -induced, 68 kDa	A
<i>TGFB2</i>	Transforming growth factor, β receptor 2 (70/80 kDa)	A
WNT/ β -catenin pathway (and related)		
<i>DKK1^c</i>	Dickkopf WNT signaling pathway inhibitor 1	C
<i>DKK2^c</i>	Dickkopf WNT signaling pathway inhibitor 2	D
<i>DKK3</i>	Dickkopf WNT signaling pathway inhibitor 3	A
<i>DKK4</i>	Dickkopf WNT signaling pathway inhibitor 4	E
<i>FZD1^c</i>	Frizzled class receptor 1	B
<i>FZD10</i>	Frizzled class receptor 10	A
<i>TCF3^c</i>	Transcription factor 3	C
<i>TCF7L1</i>	Transcription factor 7-like 1 (T-cell specific, HMG-box)	A
<i>TCF7L2</i>	Transcription factor 7-like 2 (T-cell specific, HMG-box)	A
<i>WNT5a^c</i>	Wingless-related MMTV integration site family, member 5A	C
EMT		
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4	A
<i>FN1</i>	Fibronectin 1	B
<i>SNAI1^c</i>	SNAIL family zinc finger 1	C
<i>SNAI2 (SLUG)^c</i>	SNAIL family zinc finger 2	C
<i>VIM</i>	Vimentin	A
<i>ZEB1^c</i>	Zinc finger E-box binding homeobox 1	C
<i>ZEB2</i>	Zinc finger E-box binding homeobox 2	A
Angiogenesis/endothelial		
<i>VCAM1</i>	Vascular cell adhesion molecule 1	A
<i>VWF</i>	von Willebrand factor	B

^aSelection of genes as analyzed by microarray; genes are listed alphabetically per group.

^bExpression level of the gene in the adenoma CD31⁺/CD45⁺ SP (pSP): A, when more than 1.5-fold upregulated in the pSP versus the pMP in all five of the adenoma samples; B, in four out of the five tumors; C, in three out of the five tumors; D, in two out of the five tumors; E, when more than 1.5-fold downregulated in at least three of the five tumors; and F, when there was less than 1.5-fold difference versus the MP.

^cmore than 1.5-fold upregulation but $P > 0.05$.

the pSP (Supplementary Table S8); EGFR has been found to be involved in tumor cell migration in craniopharyngiomas (Hölsken *et al.* 2011). In addition, several components that function in NOTCH, WNT, and TGF β signaling were

elevated (Table 3 and Supplementary Table S8). These pathways play a role in EMT and 'tumor stemness' but also in pituitary development (Zhu *et al.* 2007, Vankelecom 2010). In general, gene clusters associated with organ

development and differentiation processes were found to be enriched in the pSP (see [Supplementary Tables S4 and S5](#)). Finally, genes that are implicated in angiogenesis were also upregulated in the pSP versus the pMP ([Table 3](#) and [Supplementary Tables S6, S7 and S8](#)).

Differential expression as determined from the pSP microarray interrogations was verified by qRT-PCR for a number of the most interesting genes ([Fig. 1E](#)). For these analyses, the five microarrayed NF-A samples were complemented with two additional tumors (one NF-A and one GH-A; see [Table 1](#)). The results of qRT-PCR essentially confirmed the microarray data, although statistical significance was not always reached in the relatively limited number of samples analyzed thus far. All 17 genes examined showed upregulated expression in the pSP as compared with the pMP (nine with statistical significance, eight with an upward trend; [Fig. 1E](#)). Most remarkable were the elevated expression levels of *CD44*, *IL6*, *NOTCH2*, *PDGFRB*, *SNAI2*, *VIM*, and *ZEB2* in the pSP over the pMP (>55-fold). A higher expression level (>55-fold) was also found for *CXCR4*, *FN1*, *HES1*, *PDGFC*, and *TGFBR2*, in at least five out of the seven samples. The expression of *CDH1* was also checked but was not significantly different in the pSP versus the pMP, although a trend of downregulation was measured in the pSP of four out of the seven adenomas analyzed. The expression of E-cadherin, VIM, IL6, and NOTCH2 was further analyzed by immunohistochemistry in paraffin-embedded sections of pituitary adenomas that

were used for the gene expression analysis described earlier in this paper ($n=6$; see [Table 1](#) and [Supplementary Fig. S2D](#)). Tumors with high E-cadherin immunoreactivity contained some zones and cell clusters wherein E-cadherin was downregulated, and VIM was expressed in some regions or in small nests of tumor cells (in addition to the pericapillary cells), which together suggest that some of the tumor cells might have undergone EMT. IL6 immunoreactivity was observed in a couple of clustered cells (two to four per section) in three out of the six adenomas analyzed, and NOTCH2 immunoreactivity occurred in one to two cells per section in four out of the six adenomas ([Table 1](#) and [Supplementary Fig. S2D](#)). IL6 and NOTCH2 immunoreactivity were found in the same adenomas, except for PA 34.

Human pituitary tumors contain sphere-forming cells that segregate to the pSP

TSCs generate spheres ('tumorspheres') when they are brought under appropriate suspension-culture conditions ([Vankelecom & Gremeaux 2010](#), [Clevers 2011](#)). When pituitary adenoma cells were cultured in the presence of B27 and bFGF, free-floating spheres developed progressively. After 7 days of culture, bright and mostly smooth-edged spheres were obtained that clearly differed from the irregular clumps of aggregated cells that also formed (NF-A and GH-A ($n=13$); [Fig. 2A and B](#)). Sphere cells were found to express the stemness markers

Figure 2

Human pituitary tumors contain sphere-forming cells segregating to the pSP. (A) Smooth-edged spheres (arrows) develop in cultures of total pituitary adenoma cells, which are clearly distinct from clumps of cells sticking together (arrowheads). (B) Examples of spheres at higher magnification. (C) Spheres (arrow) show Sox2 immunofluorescent signal, whereas clumps do not (arrowheads). Nuclei are stained with ToPro3. A representative example is shown (left: light-microscopic image; right: overlay). (D) Secondary spheres (arrows) form after the dissociated primary

sphere cells are reseeded. (E) Example of a secondary sphere at higher magnification. (F) Third-generation spheres (arrow) develop after the dissociated second-generation sphere cells are reseeded. (G) Spheres develop from pSP cells of pituitary adenoma, although their number is low. In pMP cell cultures, only clumps of cells are observed (inset). All of the pictures were taken at 7 days of culture. Scale bars: 50 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0546>.

SOX2 and NESTIN (Chen *et al.* 2009), which thereby supported their TSC character ($n=3$; NF-A ($n=1$) and GH-A ($n=2$); Fig. 2C and Supplementary Fig. S3A and B, see section on supplementary data given at the end of this article). At the end of the culture period (days 7–10), some of the spheres contained cells that expressed the hormone produced by the original tumor, which suggests some degree of spontaneous differentiation and further supports their TSC phenotype (Supplementary Fig. S3C). To examine the self-renewal capacity of the sphere-initiating cells (as a further property of TSC), primary spheres were dissociated, and cells were seeded again. Secondary spheres with similar morphology developed, although they were fewer in number ($n=9$; Fig. 2D and E), probably because of a considerable loss of cells after the dispersion of the primary spheres. Third-generation spheres were also obtained, but these were still scarcer (Fig. 2F).

To investigate whether the sphere-forming cells belong to the pituitary tumor's SP, CD31[−]/CD45[−] SP cells were sorted from adenomas and seeded under sphere-forming conditions ($n=3$). Spheres developed in the pSP cultures (Fig. 2G) and were absent from pMP cultures, which contained only irregular clumps of cells (Fig. 2G, inset). However, only very few spheres could be obtained (two to four spheres per 20 000 pSP cells originally seeded), which made it impossible to obtain enough viable cells for further passaging after dispersion.

Together, the present findings provide supportive evidence that human pituitary adenomas contain self-renewing sphere-forming cells that give rise to spheres that express stemness markers, which are considered to be properties of TSCs. The findings further show that the sphere-initiating cells segregate to the pSP.

In addition to the *in vitro* sphere-forming assay, CSC/TSC activity is typically tested *in vivo* by (xeno-) transplantation in immunodeficient mice (Vankelecom & Gremeaux 2010, Clevers 2011, Chen *et al.* 2012, Schepers *et al.* 2012, Boumahdi *et al.* 2014). However, we observed that human pituitary adenomas, irrespective of their hormonal phenotype, do not grow (i.e., expand) in immunodeficient SCID mice. Although small tumor pieces survived for 3–4 months after s.c. implantation, no enlargement was detected (data not shown). Xenograft adenoma growth failed not only in SCID mice but also in the more immunodeficient NOD-SCID and NOD/SCID^{IL2Rγ^{−/−}} models after the s.c. implantation of the tumor parts as well as the implantation of dissociated cells (data not shown). Similarly, pituitary adenoma fragments engrafted under the kidney capsule survived but did not visibly grow in size (as analyzed after 3–6 months;

Supplementary Fig. S4, see section on supplementary data given at the end of this article). Therefore, to be able to further functionally characterize the pituitary tumor SP and to explore its TSC-associated phenotype, we turned to a well-established pituitary tumor cell line, the mouse corticotrope AtT20, which has previously been shown to grow tumors after s.c. implantation in immunodeficient mice (Taguchi *et al.* 2006).

Characterization of AtT20 SP and tumorigenic activity

First, we examined whether the AtT20 cell line contains a SP. A verapamil-blockable Hoechst^{low} cell population was detected that amounted to $1.0 \pm 0.3\%$ of the total cells ($n=12$; Fig. 3A). This AtT20 SP showed upregulated expression of the analyzed 'tumor stemness' genes *Cd44* and *Cxcr4* (Fig. 3B).

To study *in vivo* tumorigenic activity, we first verified the tumor-growing capacity of AtT20 cells in SCID mice. The s.c. injection of 5×10^5 cells resulted in a gradually developing tumor (Supplementary Fig. S5A, see section on supplementary data given at the end of this article), which was palpable after 15–20 days and further expanded in volume until the mouse became moribund, most probably as a result of corticoid overproduction because of increased adrenal stimulation by the AtT20-produced ACTH (Taguchi *et al.* 2006). A SP remained present in the AtT20-derived xenograft tumors (as analyzed after 50–55 days), although the proportion was lower than that in the cell line in culture (0.5% versus 1%; $n=3$).

SP and MP cells were then sorted by FACS from cultured AtT20 cells, and different numbers (10 000, 5000, and 1000 cells) were s.c. injected into SCID mice. Although all of the cell populations induced tumor growth, SP-derived tumors expanded faster (Supplementary Fig. S5B) and/or became larger (Fig. 3C and Supplementary Fig. S5B) as compared with MP-derived tumors. The SP cells developed a tumor with a reconstitution of the SP/MP profile (0.5% SP; $n=2$), whereas the MP cells generated tumors with a lower proportion of SP cells (0.2%; $n=2$). Together, the present results provide indications that the AtT20 SP has tumor-proliferative dominance, and that TSC (and/or TSC-like cells) are enriched, although not exclusively segregated, in the SP.

Effects of EMT regulatory pathways in the AtT20 cell line

As described earlier in the present study, the transcriptomic data of the human pituitary adenomas supported the presence of an EMT process in the (p)SP. Therefore, we tested the effects of two important EMT regulatory

Figure 3

Identification and characterization of the SP in the AtT20 pituitary tumor cell line. (A) Dot plots of dual-wavelength FACS analysis of AtT20 cells after incubation with Hoechst 33342 alone (left) or together with verapamil (right). The SP (black) and MP (white) are gated, and SP proportions are indicated. A representative example is shown. (B) *Cd44* and *Cxcr4* gene expression in the SP and MP of AtT20 cells in culture, as determined by

qRT-PCR. Bars show means of fold SP/MP $\pm$ S.E.M. ($n=3$). * $P<0.05$.

(C) Volume of xenograft tumors grown in SCID mice as measured at days 40 and 47 after s.c. injection of the indicated number of AtT20 SP cells (black bars) and MP cells (gray bars). Bars show means $\pm$ S.E.M. (five mice for 10 000 cells and four mice each for 5000 and 1000 cells; injected in two or three independent experiments; see [Supplementary Fig. S5B](#)). * $P<0.05$.

pathways (De Craene & Berx 2013), that is, the TGF β and *Cxcr4* pathways, in the AtT20 cell line. Moreover, *Cxcr4* has been identified as a marker of CSC(-like cells) in various types of cancer (Hermann et al. 2007, Vankelecom & Gremeaux 2010, Clevers 2011). As mentioned in the previous sections, its expression was upregulated in the human pituitary adenoma pSP and the AtT20 SP (Table 3; Figs 1E and 3B). It is worth noting that these studies could not be performed with primary human pituitary adenoma cells, because these cells cannot efficiently be kept in culture and do not grow (well) *in vitro* (Hofland & Lamberts 2002).

The treatment of AtT20 cells with TGF β 1 for 72 h significantly stimulated the expression of the EMT factors *Snai2* and *Vim*, and there was a clear upward trend for *Snai1* and *Cxcr4* (Fig. 4A). However, TGF β 1 treatment did not significantly enhance the motility of AtT20 cells, which is an expected functional consequence of EMT (Fig. 4B). Treatment of AtT20 cells in culture with TGF β 1 did not significantly increase the SP proportion ($1.2 \pm 0.1\%$ versus $1.0 \pm 0.3\%$ in control culture; $n=3$; $P>0.05$). Along the same lines, the pretreatment of AtT20 cells with TGF β 1 did not lead to a higher tumor-growing capacity when they were s.c. transplanted into SCID mice, either in terms of onset or in terms of progression (volume expansion) of the tumor (Supplementary Fig. S5C).

To analyze the involvement of the *Cxcr4* pathway, we treated developing AtT20 xenograft tumors in SCID mice with the *Cxcr4* antagonist AMD3100. Interestingly, tumors treated with injections of AMD3100 grew at a slower rate and remained smaller than tumors that received injections of vehicle (Fig. 4C).

We further observed that AMD3100 significantly reduced the migratory activity of AtT20 cells as analyzed

in vitro (Fig. 4B), which thus indicated an effect on EMT. However, AMD3100 treatment did not significantly affect the expression levels of the EMT-associated genes *Zeb2*, *Snai1*, *Snai2* (although a downward trend was observed), *Vim*, and *Cdh1* or of the CSC-associated marker *Cd44*, as analyzed in the xenograft tumors after 20 days of treatment (Fig. 4D). *In vitro*, 72 h AMD3100 treatment also did not affect the expression levels of these genes, and it had no significant effect (although there was a tendency towards a decrease) on the proportion of the SP ($0.5 \pm 0.3\%$ vs $0.9 \pm 0.2\%$ in controls; $n=3$; $P>0.05$). Notably, there was a small increase in *Cxcr4* expression in the AMD3100-treated xenograft tumors (Fig. 4D); the upregulation of *Cxcr4* after AMD3100 treatment has also been reported in other tumors, supposedly as a result of the adaptation of the tumor to sustained *Cxcr4* signal inhibition (Domanska et al. 2012). Together, EMT genes – at least the ones examined in the present analysis – do not seem to be involved in the AMD3100-induced inhibition of cell motility and of xenograft tumor growth (at least as examined after 20 days of treatment). To search for possible other mechanisms of tumor growth reduction, cell viability and proliferation were evaluated. Treatment of cultured AtT20 cells with AMD3100 resulted in a twofold to threefold increase in dying/dead cells (from 2–4% in controls to 8–11% after AMD3100 treatment; Fig. 4E). In analogy, AMD3100 augmented the number of AtT20 cells that went into apoptosis (about twofold; Fig. 4F). However, no difference could be detected in the number of apoptotic cells after the 20-day AMD3100 treatment *in vivo* (data not shown). The treatment of cultured AtT20 cells with AMD3100 also reduced the proportion of proliferating cells (from about 90 to 60% after 72 h; Fig. 4G).

Figure 4

Involvement of the TGF β and Cxcr4 pathways in EMT/TSC-associated properties of the AtT20 pituitary tumor cell line. (A) Gene expression in TGF β 1-treated (72 h) and untreated AtT20 cells as determined by qRT-PCR. Bars show means of fold TGF β 1/control $\pm$ s.e.m. ($n=3$). $*P<0.05$. (B) Effect of TGF β 1 and AMD3100 on the motility of AtT20 cells. Light-microscopic pictures of the AtT20 cell culture after applying a scratch 'wound' treated or not (control) with TGF β 1 or AMD3100 for the indicated time period. Representative examples are shown. Scale bar: 100 μ m. The percentage open area was determined with TScratch Software (CSELab-ETH, Zurich, Switzerland) in the cultures without compound (black bars) or with TGF β 1 (gray bars) or AMD3100 (dark gray bars). Bars show means $\pm$ s.e.m. ($n=4$). $*P<0.05$. (C) Effect of the Cxcr4-antagonist AMD3100 on AtT20 tumor growth. Immunodeficient SCID mice with growing AtT20-derived subcutaneous tumors were treated with intratumoral injections of AMD3100 (gray) or vehicle (PBS; black) starting from day 21 (arrow) after s.c. AtT20 cell implantation. Volumes of individual tumors are shown, with horizontal

lines indicating the mean for each group at each time point ($n=3$, with an initial five mice per independent experiment, a number that declined toward days 35–42 because of the gradual death of the mice). $*P<0.05$. (D) Gene expression in AMD3100-treated (20 days) and untreated AtT20 xenograft tumors as determined by qRT-PCR. Bars show means of fold AMD3100/control $\pm$ s.e.m. ($n=3$, except for *Snai1* and *Snai2*, where $n=2$). $*P<0.05$. (E) Effect of AMD3100 on AtT20 cell viability. Percentage of dying/dead AtT20 cells (as determined by Trypan blue exclusion) after treatment with AMD3100 (gray bars) or vehicle (black bars). Bars show means $\pm$ s.e.m. ($n=3$). $*P<0.05$. (F) Effect of AMD3100 on AtT20 cell apoptosis. Percentage of TUNEL $^{+}$ cells after treatment with AMD3100 (gray bars) or vehicle (black bars). Bars show means $\pm$ s.e.m. ($n=3$). $*P<0.05$. (G) Effect of AMD3100 on AtT20 cell proliferation. Percentage of Ki67 $^{+}$ AtT20 cells after treatment with AMD3100 (gray bars) or vehicle (black bars). Bars show means $\pm$ s.e.m. ($n=3$). $*P<0.05$.

From these *in vitro* data, it can be assumed that the tumor-growth-inhibitory effect of AMD3100 at least partially involves a reduction in cell viability and proliferation. From our results so far, there are no indications as of yet that EMT is also implicated.

Pituitary tumorigenesis and pituitary stem cells

In addition to the finding of SOX2 expression in tumor-sphere cells, the results of the present microarray analysis also revealed upregulated gene expression of *SOX2* in the adenoma pSP (more than 1.5-fold in three of the five

human pituitary tumors analyzed; $P<0.05$). Interestingly, the AtT20 SP also showed higher expression of *Sox2* (as compared with the MP; [Supplementary Fig. S6A](#), see section on [supplementary data](#) given at the end of this article). A similar finding was observed in an additional pituitary tumor cell line, the rat lactosomatotrope GH3, which also contains a SP ($0.5 \pm 0.05\%$; $n=18$; [Supplementary Fig. S6A and B](#)). *Sox2* is a stemness factor that also marks the recently identified stem cells of the pituitary gland ([Chen et al. 2009](#)). In general, a link may exist between CSCs/TSCs of tumors in organs and the resident stem cells, as has been recently supported by results from

studies of several cancers (Barker *et al.* 2009, Llaguno *et al.* 2009, Zhu *et al.* 2009, Mulholland *et al.* 2010, Vankelecom & Gremaux 2010, Clevers 2011, Lapouge *et al.* 2011, Chen *et al.* 2012, Schepers *et al.* 2012, Boumahdi *et al.* 2014, Vankelecom & Chen 2014, Vanner *et al.* 2014).

To explore the pituitary stem cells in connection with the gland's tumors, we turned to the *Drd2*^{-/-} mouse model (Cristina *et al.* 2006). In the pituitaries of *Drd2*^{-/-} female mice, prolactinomas gradually develop starting at 6–8 months of age, which can be detected by MRI and after necropsy (Supplementary Fig. S7A, see section on supplementary data given at the end of this article). The CD31⁻/CD45⁻ SP (pSP) proportion in the tumor-bearing AP from *Drd2*^{-/-} mice ($1.7 \pm 0.3\%$; $n=5$; Supplementary Fig. S7B) was higher than that in heterozygous *Drd2*^{+/-} ($1.4 \pm 0.6\%$; $n=2$) and WT *Drd2*^{+/+} control littermates ($1.3 \pm 0.2\%$; $n=2$). Calculated using absolute cell numbers (see the 'Materials and methods' section), the *Drd2*^{-/-} AP contains about tenfold more pSP cells than the control mice (Fig. 5A). Similarly, the absolute number of colony-forming cells (which is also often used to investigate TSC activity *in vitro*) was higher in *Drd2*^{-/-} than in *Drd2*^{+/-} pituitaries (3.9 ± 0.7 -fold; $n=3$). In addition, colonies that developed from the *Drd2*^{-/-} pituitary were generally larger in size and more smoothly shaped than were the colonies that developed from the heterozygous mice (Supplementary Fig. S7C).

We next examined the prolactinoma-bearing pituitaries of *Drd2*^{-/-} mice for Sox2⁺ cells. According to countings in dissociated APs (see the 'Materials and methods' section), the absolute number of Sox2⁺ cells was higher in the *Drd2*^{-/-} AP as compared with the WT gland (2.4 ± 0.6 -fold; $n=3$; Fig. 5B). At least part of the increase in Sox2⁺ cells is the result of higher proliferative activity. Whereas proliferating (Ki67⁺) Sox2⁺ cells were virtually absent in WT *Drd2*^{+/+} AP at the ages tested (6–12 months), double Sox2⁺/Ki67⁺ cells were more readily observed in *Drd2*^{-/-} AP (Fig. 5C). Furthermore, as has been shown previously (Chen *et al.* 2009, Fu *et al.* 2012, Gremaux *et al.* 2012), Sox2 immunoreactive signal was not only found in the expected location in the nucleus (nuclear-Sox2⁺ (nSox2⁺) cells); some cells showed Sox2⁺ signals in the cytoplasm (cytoplasmic-Sox2⁺ (cSox2⁺) cells). The *Drd2*^{-/-} cSox2⁺ cells showed a higher increase than the nSox2⁺ cells as compared with *Drd2*^{+/+} WT pituitaries (3.1 ± 0.3 - and 2.3 ± 0.7 -fold respectively; Fig. 5B and C). *In situ*, Sox2⁺ cells in the *Drd2*^{-/-} pituitary were found in the marginal zone bordering the cleft, which is not different from the WT pituitary (Fig. 5D and Supplementary Fig. S7D). On top of this location, frequent

clusters of Sox2⁺ cells were observed within the PRL⁺ tumor cell mass in the *Drd2*^{-/-} pituitary (Fig. 5D and Supplementary Fig. S7D). In the WT gland, Sox2⁺ cell clusters are also found spread within the endocrine AP parenchyma (Fig. 5D and Supplementary Fig. S7D). Double Sox2⁺/PRL⁺ cells were not detected in either of the pituitary phenotypes. Taken together, the number of Sox2⁺ cells is clearly increased in the *Drd2*^{-/-} versus the WT pituitary, but their overall appearance in the putative stem cell niches seems to be not very different.

Discussion

Adenomas represent the most frequent pathology of the pituitary gland. Despite extensive research, not much is known about the mechanisms that underlie pituitary tumor pathogenesis.

In the present study, we demonstrated the existence of a SP in different models of pituitary tumors, including human adenomas, mouse tumors, and cell lines. During the past decade, SPs have been identified in many tumor types and cell lines, and they were often found to be enriched in cells with CSC or CSC-like phenotype or activity (Patrawala *et al.* 2005, Wu & Alman 2008, Kato *et al.* 2010, Britton *et al.* 2012, Moti *et al.* 2014). The majority of these studies looked for CSCs in malignant cancers. The present study is among the first to identify a SP in benign tumors (Wu *et al.* 2007). The pituitary adenoma SP still represents a heterogeneous population, similarly to the SPs in other tumors as well as in healthy tissues (Pfister *et al.* 2005, Uezumi *et al.* 2006). In a previous study, we also detected endothelial-type cells in the SP of the normal (mouse) pituitary; further purification led to the identification of pituitary stem cells (Chen *et al.* 2009). The endothelial-type SP cells of the pituitary tumors may represent a progenitor phenotype that drives angiogenesis during tumor expansion. The presence of endothelial progenitor cells in pituitary adenomas has indeed been suggested based on the detection of CD133, CD34, NESTIN, and VEGFR2 in non-hormonal and non-folliculostellate cells of the tumors (Yunoue *et al.* 2011). Notably, the expression picture that was distilled from the total adenoma SP was found to be largely reconfirmed in the purified CD31⁻/CD45⁻ SP (pSP). The results of other studies have also indicated that the expression characteristics of minority CSC fractions can surface in still mixed populations (Eppert *et al.* 2011).

Several upregulated genes in the (p)SP were associated with a 'tumor stemness' phenotype, which has also been reported for the SP in other tumors (Patrawala *et al.* 2005,

Figure 5

Pituitary stem cells in the *Drd2*^{-/-} pituitary tumor model. (A) Fold difference in the absolute number of pSP cells in the anterior pituitary (AP) of homozygous *Drd2*^{-/-} ($n=5$) and heterozygous *Drd2*^{+/-} mice ($n=2$), relative to WT *Drd2*^{+/+} mice (set as 1). * $P<0.05$ versus *Drd2*^{+/+} mice. (B) Absolute number of Sox2⁺ cells in the AP of homozygous *Drd2*^{-/-}, heterozygous *Drd2*^{+/-} mice, and WT *Drd2*^{+/+} mice. Bars represent means $\pm$ s.e.m. ($n=3$), with an indication of the nuclear-Sox2⁺ cells (black segments) and the cytoplasmic-Sox2⁺ cells (gray segments). * $P<0.05$ versus *Drd2*^{+/+} mice (for total Sox2⁺ cell number). (C) Immunofluorescent staining of dissociated AP cells (cytospins) from WT *Drd2*^{+/+} mice (upper panel) and homozygous *Drd2*^{-/-} mice (middle and lower panels) for Sox2 (red) and Ki67 (green). Double Sox2⁺/Ki67⁺ cells are indicated (arrows). Nuclei are stained with

DAPI. Representative examples are shown (female mice, 10–12 months of age). Scale bars: 50 μ m. (D) Confocal images of pituitary vibratome sections of *Drd2*^{-/-}, *Drd2*^{+/-}, and WT *Drd2*^{+/+} mice immunofluorescently stained for Sox2 (red) and prolactin (PRL, green). Representative examples are shown (female mice, 10–13 months of age). In all of the mouse phenotypes, Sox2⁺ cells are found in the marginal cell layer along the cleft. In *Drd2*^{-/-} pituitary, Sox2⁺ cells are clustered in small groups and are often observed within the PRL⁺ tumor cell mass (arrows), but clusters of Sox2⁺ cells are also found scattered in the AP parenchyma of control *Drd2*^{+/-} and *Drd2*^{+/+} mice. Nuclei are stained with ToPro3. Scale bars: 50 μ m. Insets show a lower-magnification overview. IL, intermediate lobe. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0546>.

Wu & Alman 2008, Kato *et al.* 2010, Britton *et al.* 2012, Moti *et al.* 2014). In addition, the NOTCH, WNT, and TGF β /BMP pathways represent not only ‘(tumor) stemness’ signaling systems but also regulatory circuits that are well known for their critical role in pituitary embryonic development (Zhu *et al.* 2007, Vankelecom 2010). Moreover, the upregulated expression of *NOTCH2* might be remarkable in this sense, because this specific member of the Notch receptor family is particularly highly expressed in the progenitor cells of Rathke’s pouch, the embryonic pituitary anlage (Zhu *et al.* 2007, Vankelecom 2010). This embryonic link may be consistent with the idea that CSCs recapitulate aspects of embryonic development. Results of other studies have also indicated the involvement of NOTCH and WNT signaling in pituitary

tumorigenesis, as concluded from the transcriptomic analysis of unfractionated adenomas (Moreno *et al.* 2005). Furthermore, the upregulation of *HIF2 α* in the SP may be of importance; in other tumors, such as glioma, *HIF2 α* regulates the tumorigenic capacity of the CSC fraction (Das *et al.* 2008). Hypoxia is known to stimulate CXCR4 expression (De Craene & Berx 2013), which was indeed found to be upregulated in the pituitary tumor (p)SP. In many cancer types, CXCR4 has been advanced as a marker of CSCs, and the pathway is known to play a role in CSC activity (Hermann *et al.* 2007, Vankelecom & Gremeaux 2010, Clevers 2011). It is also notable that the EGFR pathway substrate 8 (EPS8) was also overexpressed in the pSP in the present study (four of the five adenomas, $P<0.05$; Supplementary Table S8). This oncoprotein has

previously been found to be upregulated in multiple pituitary tumor subtypes, where it mediates survival, proliferation, and tumorigenicity (Xu *et al.* 2009a). Moreover, the accompanying EGFR was also found to be upregulated and has been shown to be involved in tumor cell migration in some cancers (Hölsken *et al.* 2011). Finally, the present expression data suggest that the PI3/Akt/mTOR/PTEN pathway is also enriched in the adenoma SP (data not shown). This pathway has been proposed to play a role in pituitary tumorigenesis (Dworakowska *et al.* 2009). Moreover, CXCR4 and PI3K/Akt are interconnected; it has been shown that the paracrine/autocrine activation of CXCR4 signaling provokes the Akt-mediated improvement in survival of glioblastoma CSCs (Rubin *et al.* 2003, Gatti *et al.* 2013).

It should be noted that the expression pattern in the pSP only minimally overlaps with the set of genes (*CD133*, *NESTIN*, *CD90/THY1*, *OCT4*, *MSI*, *JAG2*, *NOTCH4*, and *DLL1*) that have recently been identified as being upregulated in candidate pituitary TSCs (so-called 'pituitary adenoma stem-like cells' (PASCs)), which were obtained by culture of sphere-like bodies (Xu *et al.* 2009b). In the present study, only *NOTCH4*, *DLL1*, and *NESTIN* were found to be overexpressed, with *NOTCH4* and *DLL1* being upregulated only in the total SP and not in the pSP. Moreover, expression levels of the multidrug transporter genes *BCRP1* (*ABCG2*) and *MDR1* (*ABCB1*) have been reported not to be upregulated in the PASCs (Xu *et al.* 2009b). These divergent results could be mainly explained by the use of cultured cells (Xu *et al.* 2009b) versus cells that were obtained directly from the tumor (the present study; further commented on in Vankelecom (2012) and Vankelecom & Chen (2014)).

In a recent study (Donangelo *et al.* 2014), candidate TSCs were proposed to exist in pituitary tumors that develop in *Rb*^{+/-} mice. These tumors differ from typical pituitary adenomas (i.e., of the AP), because they arise in the intermediate lobe (IL) of the gland. Stem cell antigen 1 (Sca1)-immunoreactive cells sorted from these IL tumors displayed higher sphere-forming capacity as compared with the Sca1⁻ cells as well as increased expression of Sox2 and nestin, and tumor-growth advantage after the injection of a large number of cells (10⁵) into the brain striatum of NOD-SCID^{IL2Rγ-/-} mice. We previously showed that pituitary 'stemness' is rather accompanied by the absence of Sca1 and that Sca1 expression is mainly found in the endothelial fraction of the SP (Chen *et al.* 2009). These findings were later supported by the detection of Sca1 in progenitor cells of endothelial or mesenchymal origin in mouse pituitary cell lines (Mitsuishi *et al.* 2013). Thus, Sca1

expression might represent a difference in the signature of the IL tumor TSCs and the normal pituitary stem cells. Alternatively, Sca1⁺ cells from the IL tumors may also include endothelial progenitor cells (CD31⁺ cells were not removed from the Sca1⁺ fraction), which would facilitate tumor xenograft development by providing angiogenic support and which could be an (additional) explanation for the tumor-growth advantage. Similar to the findings presented here the Sca1⁺ fraction did not fully retrieve the tumorigenic activity, and Sca1⁻ cells also formed tumors (although to a lower extent). Intriguingly, in the majority of the xenograft tumors derived from the Sca1⁺ cell population, Sca1⁺ cells could no longer be detected, which does not exactly fit with the definition of self-renewing CSCs. It should also be noted that Sca1 cannot be employed as a (potential) TSC marker in human pituitary adenomas, because this gene (product) does not exist in humans.

In contrast to the highly penetrant *Rb*^{+/-} IL tumors (Donangelo *et al.* 2014), benign human pituitary adenomas (transplanted s.c. or subrenally) did not expand in mice regardless of the grade of immunodeficiency of the mice. Xu *et al.* (2009b) reported 'growth and reconstitution of the original tumor' from their PASC spheroids (as analyzed for one GH-A) when they were implanted into the forebrain of NOD/SCID mice, but no clearly demarcated tumor or expansion was shown (further reviewed in Vankelecom (2012) and Vankelecom & Chen (2014)).

Because the standard xenograft assay did not work for the benign human pituitary adenoma, we further turned to *in vitro* TSC assays and to the well-established pituitary tumor AtT20 cell line. Cultured cell lines are often used as complementary paradigms for studying (candidate) CSCs. Moreover, in cell lines of multiple cancer types, the SP has been found to be enriched for authentic or candidate CSCs (Patrawala *et al.* 2005, Wu & Alman 2008, Kato *et al.* 2010, Britton *et al.* 2012). First, we discovered that human pituitary adenomas contain self-renewing sphere-forming cells that express the stemness markers SOX2 and NESTIN and that the sphere-initiating cells segregate to the pSP. In addition, the AtT20 cell line also contains a SP that was found to be enriched for cells that are more tumorigenic (i.e., grow faster and larger tumors) than MP cells, that better reconstitute the tumor's original heterogeneity (SP/MP profile) – a further property of CSCs, and that express CSC-associated genes. The segregation of tumorigenic activity in the SP is not absolute, as has also been observed in various other studies on CSCs (Boumahdi *et al.* 2014, Vanner *et al.* 2014) and in the *Rb*^{+/-} IL tumors mentioned earlier (Donangelo *et al.* 2014).

The (p)SP identified also displays an expression pattern that is supportive of EMT occurring in that tumor compartment. In recent studies, CSCs and EMT have been linked, in that EMT has been shown to act as a major driver of CSC generation and activity (Mani *et al.* 2008, Morel *et al.* 2008, Kong *et al.* 2010, Pirozzi *et al.* 2011, Yoon *et al.* 2012, De Craene & Berx 2013). In addition to being an argument in favor of the CSC(-like) phenotype of the pSP, the detection of EMT may also have implications for understanding of pituitary tumor behavior. In several cancers, EMT, or EMT-driven CSCs, are involved in expansion, invasion, resistance to hostile conditions (including therapy), and recurrence (Mani *et al.* 2008, Morel *et al.* 2008, Bertran *et al.* 2009, Kong *et al.* 2010, Pirozzi *et al.* 2011, De Craene & Berx 2013). The GO enrichment picture, which includes gene clusters associated with cell motility and migration as well as the expression of chemotactic receptors like *CXCR4* in the adenoma pSP, further supports the migratory capacity. Notably, the highest relative expression levels in the pSP (and the highest differences in the pSP versus the pMP) of the EMT-associated genes *ZEB2*, *SNAI2*, and *TWIST1* were found in the adenoma sample with the most aggressive character regarding the invasion of neighboring structures (PA 40 in Table 1). On the other hand, from the data for a limited number of tumors assessed for invasive character (i.e., PA 03, 06, 07, 10, 15, 17, 19, 20, 22, 23, 33, 34, 35, 38, and 40), no correlation emerged (yet) between invasiveness and SP proportion. The pSP compartment also expressed angiogenic factors, which could indicate that TSCs drive tumor angiogenesis to support the growth of the tumor. CSCs may also contribute to neovascularization through transdifferentiation to endothelial cells, as has been reported for glioblastomas (Dong *et al.* 2011). Processes such as EMT, angiogenesis, and hypoxia are known to contribute to the maintenance and regulation of CSCs in interaction with their microenvironment.

Because human pituitary adenomas fail to grow not only *in vivo* but also *in vitro* (Hofland & Lamberts 2002), EMT was further studied in the AtT20 cell line. The TGF β pathway is known to be an important regulator of EMT (Bertran *et al.* 2009, Pirozzi *et al.* 2011, De Craene & Berx 2013). Although TGF β activation stimulated the expression of some EMT regulatory and 'stemness'-associated genes, the treatment of AtT20 did not increase EMT-linked cell motility; neither did pretreatment induce faster or more pronounced xenograft tumor formation. Notably, the effect of the *in vitro* pretreatment might have faded away during *in vivo* growth. In general, TGF β is known to play a dual role in tumor pathogenesis: it not

only promotes tumor progression (angiogenesis, invasion, and metastasis) by stimulating tumorigenic activity and EMT (Mani *et al.* 2008, Bertran *et al.* 2009, Pirozzi *et al.* 2011, De Craene & Berx 2013) but also inhibits tumor growth by reducing cell proliferation (Ramsdell 1991, Tirino *et al.* 2013). We indeed observed a small tendency towards slower tumor growth after pretreatment of the AtT20 cells with TGF β 1 (see Supplementary Fig. S5C). Furthermore, it should be mentioned that AtT20 cells already display some mesenchymal character under basal conditions, as concluded from their spindle-shaped morphology and their basic motility in the scratch assay (data not shown), which may explain the limitation to supplementarily activate a mesenchymal phenotype in these cells. Regarding the CXCR4 pathway, which was the second EMT regulatory system that was tested, inhibition led to the reduction of AtT20 cell motility and of tumor growth. The latter finding is consistent with results from a previous study that indicated that anti-CXCR4 treatment suppressed primary brain tumor growth and that treatment of mice with AMD3100 resulted in decreased proliferation and invasion of the xenografted brain tumors as well as in better survival of the animals (Rubin *et al.* 2003). In addition, the activation of CXCR4 has been shown to stimulate cell proliferation in human pituitary adenoma cultures (Barbieri *et al.* 2008). In the present study, we showed that the Cxcr4 pathway is involved in pituitary tumor growth *in vivo*. AMD3100 was found to induce AtT20 cell death and apoptosis and to reduce cell proliferation (as analyzed *in vitro*), which is similar to findings by others for pituitary and other tumors (Barbieri *et al.* 2008, Gatti *et al.* 2013). Whether EMT is (also) implicated in the growth-inhibitory effect of AMD3100 is not yet clear; the expression of the tested selection of EMT genes was not affected after long-term treatment *in vivo* (20 days) or short-term exposure *in vitro* (72 h). EMT-associated genes other than the ones analyzed in the present study may still be involved (as described for breast CSCs; Yoon *et al.* 2012). Neither the activation of the TGF β pathway nor the inhibition of the Cxcr4 pathway significantly affected the proportion of SP cells, although the effects might be small, and the absence of an effect on proportion does not exclude a possible effect on the (molecular) activation status of the SP cells. Further studies are needed to shed more light on these mechanisms.

An important question in cancer research concerns the link between tumorigenesis (and CSCs) and the tissue's own stem cells. In several types of cancer, it has been shown that tissue stem cells are at the origin of tumors and/or of CSCs (Barker *et al.* 2009, Llaguno *et al.* 2009,

Zhu *et al.* 2009, Mulholland *et al.* 2010, Lapouge *et al.* 2011, Chen *et al.* 2012, Schepers *et al.* 2012, Boumahdi *et al.* 2014, Vanner *et al.* 2014). On the other hand, stem cells may not directly be involved; rather, they might become activated when a tumorigenic ‘assault’ occurs in their tissue (Vankelecom & Gremeaux 2010, Vankelecom & Chen 2014). In the present study, we observed that the pSP, the Sox2⁺, and the colony-forming cells were increased in number when tumors were present in the pituitary, as indicated by the results of analyses using *Drd2*^{-/-} mice, in which prolactinomas develop within their natural micro-environment. These results indicate that the pituitary stem cell compartment is activated during tumorigenesis, at least by a rise in cell number. It should be noted that the populations identified may comprise both the ‘normal’ pituitary stem cells and the (potentially stem-cell-derived) TSCs or TSC-like cells. At present, it is not possible to distinguish between these two on the basis of the properties analyzed (SP, Sox2 expression, and colony formation). The fact that cell numbers and increments are not identical among the three populations (pSP, Sox2⁺, and colony-forming) indicates that they do not fully overlap, as has also been observed previously (Chen *et al.* 2009, Fu *et al.* 2012). Moreover, more than one (cancer) stem cell population has also been detected in other tissues and tumors (see, e.g., Zhu *et al.* 2014). The finding that these populations expand in number may indicate that the stem cells react during tumorigenesis and that they might play a role in the pathogenic process. Notably, the higher increase in cSox2⁺ cells in the tumorous pituitary might support a higher differentiation rate toward tumor PRL⁺ cells (Chen *et al.* 2009, Fu *et al.* 2012, Gremeaux *et al.* 2012). In addition, Sox2⁺ cell clusters were often observed within the PRL⁺ tumor cell masses, although they were also scattered over the parenchyma in the normal AP. Whether existing clusters are entrapped during tumor formation or whether they play a more active role in the tumorigenic process, either directly (as origin) or indirectly (as tumor-inducing or tumor-tropic), needs to be further clarified (e.g., by lineage tracing). In a recent study, the results of lineage tracing did not reveal a direct link of descent between transgenically Wnt/ β -catenin-induced cranio-pharyngioma in the pituitary and the Sox2⁺ stem cells. Instead, the findings indicated that the Sox2⁺ stem cells rather acted as paracrine tumor-activating cells in this model (Andoniadou *et al.* 2013). Adamantinomatous cranio-pharyngioma is a typically pediatric tumor that probably originates from ectopic remnants of Rathke’s pouch, and it is thus different from the typical AP tumors. The transcription factor Sox2 itself may also play a role

in pituitary tumorigenesis, for example, when it is not properly shut down by the tumor suppressor p27 (at least as demonstrated for intermediate lobe tumors; reviewed by Vankelecom & Chen (2014)). In addition, Sox2 can act as an oncogene in tissues where it is expressed in the putative stem cells (e.g., lung, esophagus, and skin; Arnold *et al.* 2011, Boumahdi *et al.* 2014). Moreover, the overexpression of other pituitary stem cell factors (like Prop1) from the early Rathke’s pouch state onwards may lead to tumor formation in the gland (reviewed by Vankelecom & Chen (2014)). Again, Prop1 is not found within the tumors but in non-neoplastic regions surrounding the lesions, which indicates that Prop1 overexpression does not generate tumor-driving stem cells but rather generates trophically supporting cells.

In conclusion, we identified a SP in pituitary tumors that displays TSC-associated molecular and functional characteristics. Identification of this cell population could eventually help us to better understand pituitary tumor pathogenesis, and it may have an effect on the clinical management of the disease. The global molecular profile of the pSP may thereby serve as a valuable source for identifying potentially interesting therapeutic targets for further examination.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0546>.

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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